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Description

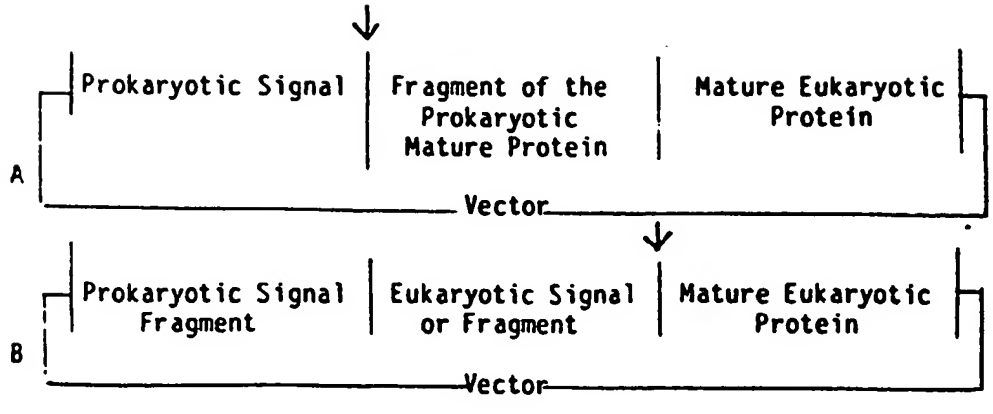
This application relates to methods for manufacturing proteins in bacteria. It particularly is concerned with isolating eukaryotic proteins from the periplasm of transformed bacteria while minimizing proteolytic degradation of the protein and contamination by nonperiplasmic proteins. This application also relates to the synthesis of mature eukaryotic proteins in bacterial hosts. It is directed to providing vectors that will express hybrid preproteins in high yields in host cells, cleave the signal sequence from the preprotein and secrete mature eukaryotic protein in the periplasmic space of the host cells.

Literature that should be consulted in regard to this application is U.S. patent 4,375,514 and 4,411,994; U.K. patent application 2,091,268A (published 1982); I. Palva et al., "Gene" 22: 229-235 (1983); H. Inouye et al., "J. Bact" 148(2): 434-439 (1983); K. Talmadge et al., "P.N.A.S. USA" 77(7): 3988-3992 (1980); K. Talmadge et al., "P.N.A.S. USA" 77(6): 3369-3373 (1980); European Patent Application 114,695; R. Picken et al., "Infection and Immunity" 42(1): 269-275 (1983); T. Silhavy et al., "Microbiological Reviews" 47(3): 313-344 (Sept. 1983); 3. Kadonaga et al., "J. Biol. Chem." 259(4): 2149-2154 (Feb. 1984); International PCT application WO 84/00774 (Mar. 1984); O. Zemel-Dreasen et al., "Gene" 27: 315-322 (1984); S. Michaelis et al., "J. Bact." 154(1): 366-374 (Apr. 1983); European Patent Application 114,695 (published Aug. 1, 1984); and G. Gray et al., "Biotechnology" pp 161-165 (Feb. 1984).

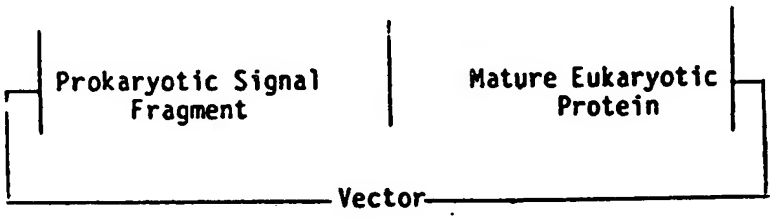
Many naturally occurring secretory and membrane proteins are initially synthesized as nascent or intracellular preproteins. These are proteins in which a "signal" polypeptide is linked to the amino acid residue that will become the amino terminus of the mature protein upon secretion. The signal polypeptide is a peptide sequence which enables the mature protein to pass through the cellular membrane of the cell. The signal peptide is cleaved away, or "processed", in passing through the cellular membrane by a mechanism that is under study. If the processing occurs properly the mature protein will be free of any amino terminal extraneous signal amino acid residues and will have the proper amino terminal amino acid. Thus, if a heterologous gene which includes the DNA encoding a signal sequence is expressed by a host gram negative bacterial cell and the signal is then cleaved properly by the host, the mature protein without an appended methionine moiety is secreted into the periplasmic space of the host, i.e., the space between the inner, or cytoplasmic, membrane and the outer membrane of the host. Also known are host-vector systems in which the signal protein and at least an amino terminal portion of the mature protein ordinarily associated with the signal is expressed and processed while linked to a heterologous protein, thereby resulting in the secretion of a fusion protein.

Secretion of mature eukaryotic protein into the periplasm of gram negative bacteria such as E. coli has been an objective of the art for a number of years. Periplasmic secretion is a desirable objective because the product is thereby compartmentalized between the inner and outer cell membranes of the culture cells and not exposed to the rigors of the extracellular medium. Exposure to these rigors, e.g., dilution, unfavorable salts or pH, oxidation, foaming, mechanical shearing and proteases, has handicapped the commercialization of nonperiplasmic secretion systems such as those using bacillus or yeast. Also, periplasmic compartmentalization simplifies recovery and purification of the desired mature eukaryotic protein.

Heretofore the vector constructions employed for periplasmic secretion in gram negative bacteria have, to applicants' knowledge, all entailed the use either of complete eukaryotic signals or of partial hybrids. Constructions which encode two typical partial hybrids are schematically shown below, with the secretion cleavage sites generally encountered being designated with an arrow.



Constructions like the partial bacterial preprotein-mature eukaryotic protein fusions shown in schematic A result in the secretion of fusions of the prokaryotic and eukaryotic proteins. It is inconvenient and sometimes impossible to remove the extraneous amino terminal amino acids encoded by the prokaryotic DNA. On the other hand, constructions like those of schematic B, result in the secretion of mature protein but yields of secreted mature eukaryotic protein are less than desired. It would be useful to construct hybrid vectors as described below which could be expressed as direct hybrids of the prokaryotic signal and mature eukaryotic protein, processed by hosts and secreted into the periplasm. Such a vector is shown below.



J. Kadonaga et al., op cit, prepared a vector of the direct hybrid type in which the beta lactamase signal was linked directly to DNA for mature chicken triose phosphate isomerase. However, the isomerase was apparently neither secreted nor processed but resided as a hybrid protein both free in the cytoplasm and attached to the cytoplasmic side of the inner membrane. These authors concluded that the amino acid sequence of at least the early part of a mature secreted bacterial protein is critical to secretion across the inner membrane of E. coli.

The reasons for the relative difficulty previously encountered in obtaining the secretion of the product of direct hybrids are unknown. However, applicants speculate that while secretion and processing machinery are conserved in bacteria (so that eukaryotic preproteins have been processed in several cases), or the machinery is somewhat flexible (as shown in processing of schematic A-type fusions), a particularly difficult obstacle to secretion would appear to be posed by constructions in which such bacteria must process the expressed product at a completely artificial cleavage site, i.e., a site which is neither a prokaryotic nor eukaryotic site for about from 1 to 3 residues on either side of the point at which hydrolysis occurs. Contrary to this expectation, and the prior failures and skepticism of those skilled in the art, applicants have found that periplasmic bacteria indeed can process direct fusions of prokaryotic signals with mature eukaryotic proteins, and can do so with secreted mature protein yields higher than those obtained by applicants with eukaryotic preproteins. Particularly satisfactory results have been obtained in secreting mammalian growth hormones.

Mammalian growth hormone is a normal product of the pituitary gland. Mammalian growth hormones are now known to exhibit a degree of species cross-specificity, a function of similar amino acid sequence and conformation. Human growth hormone (hGH) consists of 191 amino acids and has a molecular weight of about 21,500. HGH is in clinical use for the treatment of hypopituitary dwarfism. It also has been proposed to be effective in the treatment of burns, wound healing, dystrophy, bone knitting, diffuse gastric bleeding and pseudoarthrosis.

Recently, hGH has been synthesized in recombinant host cells. See, for example, U.S. 4,342,832. HGH is not significantly degraded by bacterial cells and can be produced directly if the gene for its direct expression, including the appropriately placed start codon, is linked to a suitable promoter. Because prokaryotes frequently do not remove the amino-terminal methionine from the resulting protein, expression of heterologous hGH DNA under control of a bacterial promoter as shown in U.S. Patent 4,342,832 yields hGH having methionine as its first amino acid. The reason for this is that the DNA ATG codon (start codon) is ultimately expressed as methionine. Results to date, for example, with production of hGH in E. coli, have shown that the host cell has only a limited intracellular ability to cleave methionine from hGH and only limited techniques presently exist to do so in vitro.

E.P.O. Publication Number 127305 provides for the synthesis and secretion of mature hGH in prokaryotic hosts by transforming such hosts with prehGH, i.e., with hGH having its normal eukaryotic signal sequence. Host cells were able to express prehGH, to recognize the eukaryotic signal and to process the preprotein properly. Mature hGH was then recovered from the periplasm, but yields were not as high as desired.

DNA sequences encoding a prokaryotic secreting signal sequence operably linked to the 5' end of DNA coding for eukaryotic proteins or for mature hGH are also described in the EP-A-55 942 and WO-A-84/03519

One currently used technique for recovery of periplasmic protein is called spheroplasting (H. Neu et al., 1964, "Biochem. Biophys. Res. Comm." 17: 215). This process entails the use of lysozyme to lyse the bacterial wall. It is not attractive particularly for large scale recovery of therapeutic proteins because it entails the addition of another contaminant protein to the periplasmic extract and the spheroplasts are mechanically and osmotically fragile. Further, lysozyme is relatively costly.

Another method is called osmotic shock (H. Neu et al., 1965, "J. Biol. Chem." 240(9): 3685-3692). This is disadvantageous principally because it requires two steps, first treatment of viable cells with a solution of high tonicity and second with a cold water wash of low tonicity to release the periplasmic proteins.

These methods have been practiced on viable cells. The use of viable cells is undesirable because their proteolytic enzymes are fully active. When applicants attempted to recover secreted hGH from a viable culture of E. coli, a proteolytic clip of unknown origin removed the amino terminal phenylalanine from about 10 to 20 percent of the hGH.

Accordingly, it is desirable to provide improved methods to recover periplasmic proteins. Such improved methods would minimize proteolytic degradation by proteases during recovery, and in general would be sufficiently delicate to minimize contamination of periplasmic protein by intracellular proteins. They also would permit the recovery of large proportions of periplasmic protein by manipulative steps more amenable to commercial, large scale use than currently available procedures, and would not entail the use of contaminating proteinaceous reagents.

It is also desirable to express and secrete high periplasmic amounts of mature eukaryotic protein in bacterial hosts.

It is further desirable to obtain periplasmic mature human growth hormone in elevated amounts.

In a first aspect the invention provides a hybrid DNA sequence encoding a protein having at least the amino terminal sequence of mature hGH operably linked to a DNA sequence encoding an enterotoxin signal.

DNA encoding E.coli enterotoxin signals are particularly useful in this regard. This signal DNA is characterized by not being linked (a) at its 3' end to the 5' end of DNA encoding mature enterotoxin or (b) at its 5' end to the 3' end of the enterotoxin promoter. It is conveniently employed as a cassette in the construction of enterotoxin signal-containing vectors. A preferred enterotoxin is STII.

The STII Shine-Dalgarno (S.D.) sequence is a particularly powerful ribosome binding site which contributes to yield improvement. It ordinarily is linked to prokaryotic promoters such as the tryptophan (trp) or bacterial alkaline phosphatase (AP) promoters, and could be employed with any promoter system.

In a second aspect the invention provides a replicable vector containing, in 5' to 3' order, a promoter, a Shine-Dalgarno sequence, a hybrid DNA sequence as defined above and a termination region comprising a stop codon; said promoter controlling transcription of the hybrid DNA sequence.

In a third aspect the invention provides a method comprising;

(a) constructing a vector comprising a DNA sequence encoding a protein having at least the amino terminal sequence of mature hGH operably ligated to a DNA sequence encoding an enterotoxin signal, the DNA being operably linked to the STII Shine-Dalgarno sequence;

(b) transforming a host cell capable of processing the hybrid and secreting the mature eukaryotic protein with the vector;

(c) culturing the transformed host cell; and

(d) recovering the mature protein which comprises at least the amino terminal sequence of hGH from the periplasm of the cell. Desirably said step of recovering the mature protein from the periplasmic space comprises:

(a) killing the cell;

(b) freezing the cell;

(c) thawing the cell; and

(d) separating the periplasmic proteins, including the eukaryotic protein, from the remainder of the cells.

In a fourth aspect the invention provides a prokaryotic cell culture comprising;

(a) a mature eukaryotic protein and

(b) a hybrid DNA encoding a direct fusion protein of an enterotoxin signal directly fused at its carboxyl terminus to the amino terminus of the mature eukaryotic protein; and wherein the mature protein is located in the periplasm of the cell.

In a fifth aspect the invention provides a prokaryotic cell culture comprising;

(a) mature hGH and

(b) a hybrid DNA encoding a direct fusion protein of an enterotoxin signal directly fused at its carboxyl terminus to the amino terminus of mature hGH; and wherein the mature protein is located in the periplasm of the cell. Preferably said DNA sequence encodes the full sequence of mature hGH; and said recovery step (d) comprises:

(e) killing the cell;

(f) freezing the cell; and

(g) separating periplasmic protein from the remainder of the cell.

New prokaryotic cell cultures are produced upon transformation and culture of host cells using the above method. These cultures comprise (a) mature eukaryotic protein and (b) a direct hybrid fusion protein of the mature eukaryotic protein with a prokaryotic secretion signal sequence. Ordinarily, greater than about 25 percent, generally up to about 90 percent, of the total weight of mature and fusion protein is mature protein located in the periplasm of the cell.

The preferred method comprises obtaining viable or killed cells which have been transformed to secrete a heterologous or eukaryotic protein, causing the outer membrane of the cells to become permeable for passage of the protein out through the membrane as for example by freezing and thawing, and separating the periplasmic proteins, including secreted eukaryotic protein, from the remainder of the cells. It also is advantageous to culture the cell under phosphate-limiting conditions to engender changes in the cell membranes which enhance recovery of the periplasmic protein.

A novel killing method is provided wherein transformed cells are contacted with an alkanol and heated. These cells then are treated by a cold shock method comprising freezing and thawing which enables recovery of periplasmic proteins.

These methods are employed to particular advantage in recovering hGH from gram negative organisms which express and process a direct hybrid fusion of the E. coli STII enterotoxin signal with mature hGH.

Brief Description of the Drawings

Fig. 1 discloses the STII gene, including its translated and untranslated regions. The principal portion of its S.D. sequence is overlined at nucleotides 155-161. The imputed amino acid sequence for the STII signal is located at residues -23 to -1 and for the mature STII enterotoxin at residues 1-48. Fig. 1 also discloses the processing site for STII (designated "cleavage site") and various restriction enzyme sites. The asterisk designates the likely mRNA synthesis initiation site assuming that the STII promoter includes the overlined structures at position 84 - 89 and 108 - 114.

Figs. 2a - 2d disclose the construction of a vector (ptrp-STII-hGH) encoding a secretable STII-hGH fusion protein under the control of the trp promoter and containing an STII S.D. sequence.

Fig. 3 is a detail of the plasmid tr-STII-hGH.

Fig. 3a is the nucleotide sequence of the trp promoter region, STII signal and the hGH gene in ptrp-STII-hGH.

Figs. 4a-4c disclose the construction of the vectors pAP-1 and pAP-STII-hGH encoding secretable AP-hGH and STII-hGH fusion proteins under the control of the AP promoter, the vector encoding the latter containing an STII S.D. sequence.

Fig. 5 is the nucleotide sequence of the AP promoter region, STII signal and the hGH gene in pAP-STII-hGH.

Fig. 6 is the nucleotide sequence of the AP promoter region, the AP signal and the hGH gene in pAP-1.

Detailed Description

A heterologous protein is a protein not ordinarily secreted by the bacterial cell. While such a protein may be an ordinarily intracellular protein of the transformed cell that has been engineered for secretion, or it may be a protein encoded by DNA from another microbe, ordinarily it is a eukaryotic protein, a fragment thereof or a fusion thereof with a prokaryotic protein or fragment. Preferably the periplasmic protein is a mature eukaryotic protein such as a hormone, e.g., hGH, an interferon or a lymphokine.

The cells to be treated herein may be obtained from cultures of transformed bacteria grown in conventional media or media tailored to the vectors or mutant host transformants used to secrete the heterologous protein.

Applicants have demonstrated the secretion of direct hybrid fusions of a prokaryotic signal and desired eukaryotic protein, notwithstanding the complexity of the host vector systems at both the level of preprotein synthesis and secretion. Plasmids were constructed in which the trp or AP promoters were employed with various directly linked prokaryotic signal and eukaryotic protein sequences. Suitable hosts were transformed with each of these plasmids and the amount and distribution of product between the periplasm and cytoplasm determined. These experiments demonstrated that mature eukaryotic proteins are secreted and correctly processed into the periplasmic space of organisms from direct hybrid fusion proteins. The following table shows the results of successful experiments. The results obtained by using the normal hGH eukaryotic signal are shown for comparison.

Table 1

Effect of Promoters and Signal Peptides on Expression and Secretion of Eukaryotic Proteins

Promoter	Signal	Gene	Host	Locations/Levels[3]	Processing[4]	Media
trp	STII	hGH	294 or W3110[8]	90 percent periplasmic; 1 gram	correct	defined or LB
AP	STII	hGH	W3110	50 percent periplasmic; 0.5 gram	correct	defined-pi[1]
AP	AP	hGH	294[2]	90 percent periplasmic; 0.1 gram	correct	LB-pi
trp	hGH	hGH	294 or W3110	90 percent periplasmic; 50 mg	correct	defined/LB
trp	STII	hLIF-A[6]	294	50 percent periplasmic; 1.9 mg	correct	LB
trp	STII	mIgG-K[7]	294	60 percent periplasmic; ND	ND[5]	LB

1 inorganic phosphate depleted medium

2 E. coli ATCC 31446

3 Variation is typically encountered among experiments. Relevant parameters include culture density, the time at which secreted protein is recovered, and other variables. The total amount of mature and fused eukaryotic protein, as well as the periplasmic percentage, must be considered approximate. The levels are reported as grams/50 equivalent culture OD units at 550 nm/liter. Cultures were grown in 10 or 20 ml fermentations in shake flasks.

4 Correct processing means that the secreted protein exhibited the same amino terminus as is found when the protein is

isolated from natural sources.

5 ND means not done.

6 human leukocyte interferon - A

7 The light chain of a murine monoclonal anti-CEA immunoglobulin.

8 E. coli ATCC 27325

Applicants' initial attempts to secrete some mature eukaryotic proteins using prokaryotic signals in the above structural format did not result in synthesis of protein in some cases, and in others expression was not accompanied by secretion. Cultures transformed with vectors for the murine immunoglobulin heavy chain (using the STII signal), human tissue plasminogen activator (using the AP promoter and signal) or a bovine prorennin (using the trp promoter and the STII signal in E. coli hosts), or hGH (using the Pseudomonas aeruginosa enterotoxin A signal) synthesized very low or undetectable quantities of either the preprotein or secreted mature protein. Other transformed cultures failed to process STII signal fusions with bovine gamma interferon, prorelaxin, interleukin-2 or bovine prorennin. These results were obtained in limited and preliminary experiments.

It is clear from this work that direct hybrid fusions of prokaryotic signals with mature eukaryotic proteins are recognized by bacterial cells, i.e., processed and transported into the periplasm. Given that knowledge, the skilled artisan must nonetheless exert diligence in identifying functional constructions.

This effort should be directed first at screening vectors encoding direct hybrid fusions with signals obtained from a variety of periplasmic bacterial proteins, e.g. enzymes or enterotoxins. If the secretion of mature eukaryotic protein is not obtained upon the transformation and culture of E. coli with such constructions then other genera of gram negative bacteria should be screened for the ability to secrete the mature protein. Lastly, the signal peptide may be mutated in order to enhance or modify its processing characteristics.

The method herein is facilitated by the ability of gram negative bacteria to recognize direct fusions with the STII enterotoxin signal peptide. Furthermore, it is based on the additional discovery that elevated yields are obtained by use of the STII S.D. sequence.

Partial amino acid sequences for hGH and STII enterotoxin preproteins are shown below with the starting amino acid for each mature protein being underlined.

hGH ... leu gln glu gly ser ala phe pro ala met ser leu...
STII ... ala thr asn ala tyr ala ser thr gln ser asn lys...

As can be seen, virtually no homology whatever exists between these two sequences in the vicinity of their signal cleavage sites. Thus it was surprising that the host cells were able to recognize the STII-hGH hybrid junction and process the STII-hGH preprotein correctly and secrete hGH.

The prokaryotic signal sequence to be used herein is the signal sequence from any bacterial secreted or cell membrane enterotoxin, or mutation thereof. The preferred embodiments are found among the heat stable (ST) and heat labile (LT) enterotoxins of E. coli. Of the ST enterotoxins, STII is most preferred.

According to Picken et al., op cit, the STII signal polypeptide was either the amino acid sequence NH_2-met lys lys asn ile ala phe leu leu ala ser met phe val phe ser ile ala thr asn ala, or this sequence with an additional carboxyl-terminal tyr ala. In fact, E. coli cleaves the signal after tyr ala. Thus, the STII signal DNA which is used in the vectors further described herein will encode the tyr ala alternative.

The prokaryotic signal may be mutated in order to increase the proportion of eukaryotic protein that is secreted. Generally, mutations in codons encoding amino acid positions outside of the hydrophobic core of the signal, e.g., residues -1, -2 or -3, are more likely to exert a significant effect on signal cleavage. The mutated DNA will express an amino acid at the site of mutation which is different from the wild type signal. Most conveniently a plurality of codons are substituted at a given position, each of which encodes a different amino acid. This is accomplished by methods known in the art. For example see S. Michaelis, op. cit., as applied to the alkaline phosphatase signal. Each individual construction then is ligated to DNA encoding the mature human protein in an expression vector, the vector used to transform hosts, the transformants cultured and the secretion level determined for each mutant as is more fully described below. Transformants that secrete optimal levels of desired protein are identified and the responsible constructions selected. With respect to the STII signal, it is expected that amino acid substitutions in the hydrophobic

region (residues -5 to -17 in Fig. 1) will not adversely affect the signal efficacy so long as the substituted amino acids are uncharged and, preferably, hydrophobic. Also, deletions or insertions of one or two like residues in this region are acceptable. The most sensitive region of the leader is residues -1 and -21 to -22 (Fig. 1). Mutations in these residues, including insertions or deletions generally are deleterious, but occasionally beneficial effects on yield or secretion are obtained. Mutations that are so extensive as to convert the expressed signal to the eukaryotic signal ordinarily associated with the desired protein or other eukaryotic proteins are not prokaryotic signal mutations as defined herein.

Mutagenic optimization is particularly desirable upon switching from one host to another while using vectors containing substantially the same signal. The reason for this is that allelic variants in the bacterial enzyme system responsible for cleaving the signal-eukaryotic protein fusion and transporting the mature protein may more readily recognize a suitably modified prokaryotic signal.

In accordance with this invention any eukaryotic protein, mutant or derivative may be secreted if proper gram negative hosts and signals are selected, provided of course that the protein per se is capable of being expressed in gram negative hosts. Such eukaryotic proteins include lymphokines, immunoglobulins and hormones. The eukaryotic proteins used herein ordinarily are mammalian proteins such as those of bovine, porcine and human origin. Particularly advantageous results are obtained with growth hormones such as human growth hormone.

Vectors for transforming hosts to express direct hybrid fusion proteins are made in conventional fashion by methods generally known to those skilled in the art. In such vectors, DNA encoding the prokaryotic signal is directly linked to DNA encoding the desired protein. This means that the signal DNA encoding the amino acid immediately upstream from the normal bacterial cleavage site is linked directly to the DNA encoding the first amino acid of the desired mature eukaryotic protein, without any intervening residual sequences from the mature prokaryotic protein or the normal eukaryotic pre sequence. Such direct linkage is accomplished by known methods such as the M13 deletional mutagensis precedure described in the examples. Alternately, one may chemically synthesize DNA encoding the signal and cleavage region. This DNA is blunt end ligated, or ligated through a convenient restriction enzyme site, to DNA encoding the remainder of the eukaryotic protein.

DNA encoding STII signal is directly linked in either fashion to DNA encoding the desired mature eukaryotic protein. When the eukaryotic protein is a growth hormone, the signal will be linked to DNA having as its two first 5' codons, codons which encode at least the first two amino-terminal amino acids of hGH, i.e., NH_2-phe pro, and preferably encode the first 15 amino-terminal amino acids of hGH. This sequence ordinarily is part of a DNA sequence encoding growth hormone such as hGH, bovine growth hormone (having the amino-terminal sequence phe pro ala met ser leu) or porcine growth hormone (having the amino-terminal sequence phe pro ala met pro leu) and the allelic variants thereof.

Suitable vectors for use herein are constructed by operably ligating the direct hybrid fusion DNA to replication and translation effecting sequences. Sequences which effect translation of the mRNA include an S.D. sequence present upstream from the prokaryotic signal. Preferably, the S.D. sequence used herein is that of STII, and it is spaced from the prokaryotic signal start codon by the natural intervening sequence (TTTT) found in the STII gene. This construction is obtained most easily by isolating the STII signal complete with its S.D. sequence and normal intervening sequence from a source of the STII gene as is further described below. However, because this portion of the STII gene is only 80 bp long it also is practical to simply synthesize the required sequence by known chemical methods. This is especially preferred if extensive mutagenesis of the signal is contemplated. The construction shown in Fig. 3a contains two S.D. sequences, one upstream S.D. sequence donated by the trp promoter and then the STII S.D. sequence in its normal relationship with the STII signal DNA. We believe that the upstream, non-STII S.D. sequence is not required. Other prokaryotic signal sequences also may be synthesized by chemical methods as they tend to be less than 100 bp in length.

Transcription of the foregoing S.D.-signal-protein sequence is under the control of a promoter. The promoter is preferably a prokaryotic promoter other than the promoter ordinarily associated with the selected prokaryotic signal. The preferred embodiment is the AP promoter, although others such as the tac, trp or lactose (D. Goeddel et al., "Nature" 281: 544 [1979]) promoters are satisfactory. While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebenlist et al., 1980, "Cell" 20: 269). The promoter does not appear to affect the proportion of eukaryotic protein that is secreted. However, it is desirable to screen combinations of promoters and signal sequences for optimal expression since both elements interact in affecting expression levels. For example, compare the results in Table 1 when the STII signal is substituted for the AP signal in combination with the AP promoter.

The promoter-S.D.-signal sequences are present in vectors containing replicon sequences compatible with the host cells. The vectors are generally plasmids rather than phage. They contain a replication site as well as phenotypic marking sequences to facilitate identification of transformed cells. For example, E. coli is typically transformed with a derivative of pBR322, (Bolivar et al., "Gene" 2: 95 [1977]), a plasmid containing genes for ampicillin and tetracycline resistance.

When a DNA sequence is "operably ligated or linked" to another it means that the DNA sequence in question exerts an influence on other DNA sequences. This generally means that the first DNA controls either the transcription or ultimately the translation of the DNA to which it is operably ligated, or affects the processing of the translated protein. For example, a promoter is operably ligated to DNA encoding a preprotein when it affects the rate of translation of the preprotein mRNA. A signal polypeptide is operably ligated to DNA encoding a desired protein when it is placed in reading frame with and ligated directly to the DNA encoding the protein. The term "ligated" in reference to a composition should not be inferred to mean that the composition is only defined in terms of its possible manufacture by ligation. On the contrary, operably lighted elements in a plasmid can be synthesized chemically as a unitary entity.

The host cells that are transformed with the foregoing vectors are bacteria (a) having a periplasmic space between two cell membranes, (b) in which the vector replicates and (c) in which the preprotein is both expressed and processed. The hosts are generally gram negative organisms, particularly Enterobacteriaceae or Pseudomonas and mutants thereof. Host vector systems (hosts transformed with vectors) are preferred in which the promoter controlling expression of the hybrid fusion is constitutively activated or derepressed. A constitutive mutant is one that is capable of secreting a given protein, which here is the promoted direct hybrid fusion and, in some cases, the normally promoted protein, without induction or other changes in culture conditions calculated to derepress or activate the promoter. Either the host cell or the promoter, or both, may be mutated in order for the host-vector system to be constitutively promoted. The promoter used in the vector may be mutated so that it is no longer capable of being repressed by a repressor protein. Such mutations are known. Alternatively, the host cells are mutated to become constitutive for proteins under the control of a wild-type or unmutated promoter. Such hosts are conveniently prepared by transduction from publically available strains containing the mutant alleles. Preferably, host cells transformed with AP promoter-bearing vectors carry a phoT or phoR mutant allele. The former is a disabling mutant believed to be in the gene encoding a phosphate ion transport protein. The phoR mutant is a disabling mutant in the gene encoding a repressor protein. These mutant alleles are widely known and available in the art and may be transfered into hosts by known techniques. Surprisingly, the constitutive hosts processed a greater percentage of the expressed product than the wild type hosts which were induced by phosphate depletion.

The host cells may, but need not constitutively express the host protein normally promoted by the promoter used to control transcription of DNA for the direct hybrid fusion. For example, either an E. coli phoA deletion mutant, which fails to express active AP, or an AP-synthesizing cell may be used as host; the active secretion of AP by the cells does not necessarily interfere with secretion of the eukaryotic protein. Ordinarily, the host bacterium will be the same species from which the signal was obtained.

While any culture medium ordinarily used for the host cells is satisfactory for the transformants, the composition of host culture medium exerts a strong effect on the secretion of eukaryotic protein by wild type hosts (rather than strain W3110 phoA, phoT or phoA, phoR). Media containing yeast extract results in improved secretion when compared to tryptone (a tryptic digest of casein) or a synthetic mixture of 19 amino acids. It appears that one or more components of yeast extract helps to activate secretion.

In the preferred embodiment the cells are to be grown under phosphate limiting conditions prior to harvest of the culture. This is the case whether or not the organism is transformed with a vector encoding a eukaryotic protein under the control of a promoter for a phosphate processing protein, or whether the cell is defective in transporting or catabolizing phosphate nutrients. Surprisingly, the use of a phosphate-limiting culture medium prior to cell harvest during the late stages of fermentation greatly improves the mechanical characteristics of transformed gram negative organisms. These characteristics enhance the efficacy of the cold shock extraction method described infra. The medium is made phosphate limiting, for example, by either precipitating the phosphate from the medium, by introducing phosphate to the culture at a limiting feed rate, or by providing an initial supply of phosphate that will be inadequate to support optimal growth of the culture beyond a certain predetermined density. The initial supply will be sufficient to support optimal cell growth during early stages of the fermentation, for example log phase growth or to an OD_{550} of about 45. The amount of phosphate will be tailored to the other nutrients and to the strain of organism used in the culture. Ordinarily, about 2.5 g/l of potassium/sodium phosphate salts are employed in media for use with E. coli W3110 (ATCC 27325), but larger amounts (about 4.0 g/l) are used with cells having phosphate metabolism defects such as the phoT mutant. This is in contrast with nonlimiting quantities of phosphate

(about 9.0 g/l). The amount of phosphate ion in the limiting culture medium at the point the culture becomes limiting is less than about 1 mM. Typically the cells are cultured under these limiting conditions for at least about 1 hour prior to harvest.

Preferably the cells are permitted to accumulate periplasmic heterologous protein to a maximum level, usually during a growth stage following logarithmic growth, prior to initiation of the treatment provided herein. The cells should be killed as soon as this point is reached or shortly thereafter. The term "killed" means that the cells at least are unable to replicate. It is likely, however, that treatment with alkanol and heat as is further described infra interdicts or destroys metabolic functions not directly associated with replication. However, other than elimination of a deleterious proteolytic activity of E. coli towards periplasmic hGH the impacted metabolic functions remain unknown. The killing procedure should not rupture, lyse or weaken the inner cell membrane of the host organism.

Cells that have reached the desired point of growth are preferably killed by immediately contacting them with an alkanol and heating. The alkanol should not be an alkanol which is lytic for cell membrines, e.g. 1-octanol. Generally, suitable alkanols include lower (C_2 to C_4) monohydroxy alkanols such as 1-butanol or ethanol, preferably 1-butanol. The alcohol should be contacted with the cells by adding the alkanol to the fermentation culture while continuously mixing.

The amount of alcohol which is added will be such as to bring the culture to an alkanol concentration of about from 0.5 percent to 10 percent vol/vol, and preferably about to 1.5 percent vol/vol for 1-butanol. Butanol is preferred because as little as about 0.5 percent can be used; larger proportions are needed for equivalent inactivation when using a propanol or ethanol.

Contemporaneous with or after the addition of alkanol to the cell culture, the temperature of the culture is increased to and held at a level sufficient in conjunction with alkanol to kill the cells. Ordinarily this will be about from 0.5 to 20 minutes at a temperature of about from 55 °C to 35 °C, the higher temperatures being employed for the lesser periods. The alkanol enables the heating to be conducted at a lower temperature than would otherwise be required, an advantage in preserving the activity of proteins to be recovered.

Cell killing is not critical. Since it is useful in preventing or retarding product degradation it may be dispensed with if the cells can be processed quickly and regulatory requirements for handling recombinant cells can be otherwise complied with. However, we have found that killing the cells approximately doubles the product protein recovery without reducing the purity of the product protein in the recovered supernatants.

After the cells are cultured and/or killed, periplasmic protein is recovered. This generally entails forming a paste of the cells, freezing the cells, thawing the cells, suspending them in buffer, and separating the periplasmic proteins from cell debris, e.g. by low speed centrifugation or filtration. The periplasmic proteins, including secreted eukaryotic proteins, are located in the supernatant. Eukaryotic periplasmic proteins may be obtained in higher specific activity, i.e. purity, than is attained with osmotic shock methods, and treatment with a hypertonic agent such as 20 percent sucrose is not required, but it should be understood that any method for causing the outer membrane of the cell to become permeable to the periplasmic protein can be used with killed cells.

The cell paste to be frozen is produced by centrifuging or filtering the cell culture and recovering the cell mass. This paste typically contains residual quantities of the fermented culture medium, e.g. LB (Luria Broth) medium; it is unnecessary to wash the cells with a freezing menstruum prior to the subsequent steps. It is not critical that a cell paste be formed as the cold shock extraction method herein is largely independent of cell density. However, the economics of freezing and thawing large volumes of material are such that small paste volumes are preferred.

The cell freezing should be as soon as possible. Generally the paste at room temperature is placed in a freezer at -20 °C until frozen, and then stored at -80 °C until further processing is required.

The frozen paste is thawed and diluted into several volumes of water or aqueous buffer, preferably about 3 or greater volumes of 10 mM tris-HCl buffer at pH8. This buffer is hypotonic to the intracellular contents of the host organisms. The following steps are performed at about 4 °C. The dilutions generally should be about 3 volumes of buffer, although the identity, concentration, and pH of buffer will vary depending upon the periplasmic protein to be recovered.

The cells are thoroughly suspended in the buffer. This step is facilitated by suspending the cells in the buffer by use of a homogenizer set at a speed at which no substantial lysis of the cells occurs.

The suspended cells are gently stirred for about from 10 to 60 minutes, ordinarily about 30 minutes. Then the cell debris and cells (containing cytoplasmic proteins) are removed by centrifugation, typically 12,000 x g for 30 minutes, or by filtration. The supernatant contains the periplasmic proteins, solubilized outer membrane proteins, residual culture medium and extracellular proteins, and a small proportion of soluble intracellular proteins. The desired eukaryotic protein may be purified from the supernatant in accord

with further procedures as desired.

The freeze-thaw extraction method described above offers considerable advantages in terms of yield and, surprisingly, purity of the desired periplasmic protein when compared to extraction of either fresh killed or unkilled transformants with buffer alone. Approximately 5 to 26 times as much hGH is recovered from frozen unkilled cells than from unfrozen, unkilled buffer-extracted cells. The supernatants from frozen killed cells were about 19 percent hGH by weight of total protein, but from fresh killed cells only about 16 percent hGH.

The foregoing method is applied in the following Examples to the recovery of hGH from E. coli transformed with a vector which expresses mature hGH as a direct hybrid fusion with the E. coli heat stable enterotoxin STII signal peptide. However, it will be appreciated that the recovery method herein is applicable to the separation of periplasmic proteins from transformed bacterial cells in general, e.g. those disclosed in U.S. patents 4,411,994 and 4,375,514; K. Talmadge et al., op cit (both citations); O. Zemel-Dreasen et al., op cit and G. Gray et al., op cit.

In order to simplify the Examples certain frequently occurring and well-known methods employed in recombinant constructions will be referenced by shorthand phrases or designations.

Plasmids are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids or sources of DNA herein are commercially available, are publically available on an unrestricted basis, or can be constructed from available plasmids or polynucleotides in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan since the plasmids generally only function as replication vehicles for the preprotein and its control sequences, or for elements thereof in intermediate constructions.

"Digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific is called a restriction site. "Partial" digestion refers to incomplete digestion by a restriction enzyme, i.e., conditions are chosen that result in cleavage of some but not all of the sites for a given restriction endonuclease in a DNA substrate.

The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements as established by the enzyme suppliers were used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters and then, generally, a number representing the microorganism from which each restriction enzyme originally was obtained. In general, about 1 µg of plasmid or DNA fragment is used with about 1 unit of enzyme in about 20 µl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop upon ligation (described below) that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis et al., 1982, Molecular Cloning pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide gel electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the DNA from the gel, generally by electroelution. This procedure is known generally. For example, see A. Lawn et al, 1981, "Nucleic Acids Res." 9: 6103-6114, and D. Goeddel et al., 1980, "Nucleic Acids Res." 8: 4057.

"Southern Analysis" is a method by which the presence of DNA sequences in a digest or DNA-containing composition is confirmed by hybridization to a known, labelled oligonucleotide or DNA fragment. For the purposes herein, unless otherwise provided, Southern analysis shall mean separation of digests on 1 percent agarose, denaturation and transfer to nitrocellulose by the method of E. Southern, 1975, "J. Mol. Biol." 98: 503-517, and hybridization as described by T Maniatis et al., 1978 "Cell" 15: 687-701.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or chromosomal integrant. Unless otherwise provided, the method used herein for transformation of E. coli is the $CaCl_2$ method of Mandel et al., 1970, "J. Mol. Biol." 53: 154.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (T. Maniatis et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately

equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis et al., Id. p. 90., may be used.

"Oligonucleotides" are short length single or double stranded polydeoxynucleotides which are chemically synthesized by known methods and then purified on polyacrylamide gels.

All literature citations are expressly incorporated by reference.

Example 1

Construction of a Plasmid Encoding for the E. coli Heat-Stable Enterotoxin (STII) Gene Signal Peptide Sequence.

The following construction is illustrated in Fig. 2a. The plasmid pWM501 (Picken et al, op cit) contains the heat-stable enterotoxin (STII) gene. A portion of the DNA which encodes the STII gene was recovered from pWM501 1 (stippled region of Fig. 2a) using the following steps. pWM501 was digested with RsaI and the 550 bp DNA fragment 2 was isolated. This gene fragment was ligated to the phage M13mp8 (J. Messing et al. in the Third Cleveland Symposium on Macromolecules: Recombinant DNA, Ed. A. Walton, Elsevier, Amsterdam (1981) pp 143-153) that had been previously digested with SmaI. The ligated DNA was used to transform E. coli JM101, a commercially available strain for use with the M13 phage. Clear plaques were recovered. The double stranded M13mp8 STII Rsa derivative 3 was isolated from an E. coli JM101 infected with this phage using standard procedures (J. Messing et al. op cit). By the use of the M13mp8 subcloning procedure just described the approximately 550 base pair fragment 2 containing the STII leader gene is now bounded by a series of different restriction endonuclease sites provided by the phage. The M13mp8 STII Rsa derivative 3 then was digested with EcoRI and Pst I and a DNA fragment 4 slightly larger than fragment 2 was isolated.

EcoRI-PstI fragment 4 was subcloned into pBR322. This was accomplished by digesting pBR322 with EcoRI and PstI and isolating the vector 5. The isolated vector 5 was ligated to the EcoRI-PstI DNA fragment 4. This DNA mixture was used to transform E. coli 294 and tetracycline resistant colonies selected. A plasmid 6 was isolated from a resistant E. coli colony and designated pSTII partial.

Example 2

Construction of a Plasmid encoding the STII Signal Peptide Under the Control of the Trp Promoter.

This construction method is shown in Fig. 2b. pSTII partial from Example 1 was digested with MnlI and BamHI and a 180 bp fragment 7 containing the STII S.D. sequence, the STII signal sequence, and the first 30 codons of the mature STII gene was isolated. DNA fragment 7 was ligated to a plasmid containing the trp promoter. One such plasmid pHGH207-1, 8, has been described previously (H. de Boer et al., 1982, in: Promoters: Structure and Function, Eds. R. Rodreguez et al. Chamberlin, Praeger Pub., New York, NY, pp 462-481). A derivative of this plasmid, pHGH207-1*, wherein the EcoRI site 5' to the trp promotor had been converted to EcoRI* by filling in with DNA polymerase I (DNA pol I) and joining the blunt ends by ligation (S. Cabilly et al., 1984, "Proc. Natl. Acad. Sci. USA" 81: 3273-3277) was used in this example. The trp promoter-containing plasmid was digested with XbaI and treated with DNA pol I and all four dNTPs to fill in the protruding sequence. The DNA preparation was then digested with BamHI and the vector containing fragment 9 isolated. Vector fragment 9 then was ligated to the 180 bp STII signal-containing DNA fragment 7 isolated above. The ligation mixture was used to transform E. coli 294 to ampicillin resistance. A plasmid designated STII leader 10 was isolated from an ampicillin resistant colony. This plasmid contains the STII signal sequence and a portion of the gene encoding mature STII under the control of the trp promoter. In the following example, the DNA sequence encoding mature hGH was operably ligated downstream from the trp - STII signal sequence.

Example 3

Construction of an Expression and Secretion Plasmid for hGH

Refer to Figs. 2c-2d for a schematic display of this method. STII leader 10 was digested with BglII then treated with DNA pol I and all four NTP's to fill in the protruding end, and then digested with BamHI. The vector-containing fragment 11 was isolated. The plasmid pHGH207-1 from Example 2 was digested with

EcoRI, treated with DNA pol I and all four NTP's to fill in the protruding end, and then digested with BamHI. The hGH gene-containing fragment 12 of about 920 bp was isolated from the BamHI digestion. These two fragments were ligated and the DNA mixture used to transform E. coli 294 to tetracycline resistance. A plasmid designated ptrpSTII-HGH-fusion 13 was isolated from the resistant E. coli colonies. This plasmid still contains extraneous nucleotides encoding a portion of the STII mature protein between the STII leader peptide sequence and the hGH structural gene. These nucleotides were deleted using the M13 site specific mutagenesis procedure (J.P. Adelman et al, 1983, "DNA" 2: 183-193).

The gene from ptrpSTII HGH fusion 13 was incorporated into the single stranded phage M13mp10 (J. Messing et al., op cit. and J. Adelman et al. op cit.). M13 mutagenesis phage and E. coli strains are commercially available. Mutagenesis was accomplished first by digesting plasmid 13 with XbaI and BamHI and then recovering fragment 14. M13mp10 was digested with XbaI and BamHI and the phage fragment (not shown) was isolated. Fragment 14 was ligated into the phage fragment and the ligation mixtures used to transform JM101. The transformed culture was plated and incubated. Phage having the fragment 14 insert were identified as clear rather than blue plaques in the E. coli chromogenic indicator lawn. Corresponding phage were grown on E. coli JM101, and the culture centrifuged. Single stranded phage 15 are present in the supernatant. Single stranded phage 15 DNA was prepared, annealled to the synthetic oligonucleotide primer 5'pCAAATGCCTATGCATTCCCAACTATACC-OH3', primer extended with DNA pol I and the four NTPs to obtain double stranded DNA (one of which strands contained the deletion), treated with T4 ligase, extracted and used to transform E. coli JM101 (See J. Adelman et al., op cit). Note that the first 14 nucleotides of the primer are the coding sequence for the 3' end of the STII signal, while the last 14 nucleotides are the coding sequence for the 5' end of the mature hGH gene. Double stranded phage were obtained from the cellular contents of the transformed JM101, transfected into plated E. coli JM101, transfer filter impressions taken of the plates and double stranded phage containing the deletion were identified on the filters by Southern analysis with a 5'-32P-labelled obigonucleotide having the DNA sequence of the primer. Double stranded DNA 17 was prepared from the E. coli infected with the M13mp10 containing the gene deletion. This DNA was digested with XbaI and BamHI and the DNA fragment 17 isolated. This was ligated in the presence of fragment 18 from similarly digested and isolated pHGH207-1. The ligation was used to transform E. coli 294 to tetracycline resistance. Plasmid ptrp-STII-HGH 19 was recovered and its nucleotide sequence determined. A detailed restriction map of this plasmid is shown in figure 3. The DNA sequence of this plasmid in the vicinity of the hGH gene is shown in Fig 3a.

Example 4

Expression and Secretion of hGH

HGH was synthesized in shake culture using plasmid 19 from Example 3. E. coli 294 was transformed with plasmid 19 and innoculated into 10-20 ml of LB medium with 5 µg/ml tetracycline in a 50 or 125 ml shake flask. The flask was cultured for 12-24 hours at 37°C without the addition of any further medium, after which the cells were recovered by centrifuging. Total cellular hGH was assayed by radioimmunoassay of sonicated cells. The secreted hGH was recovered through osmotic shock and determined to be mature hGH by SDS-PAGE and N-terminal amino acid terminal sequencing. Amounts recovered were about ten times that which is expressed when using the human hGH signal under control of the trp promoter.

Example 5

Construction of Plasmid pAP-1 Designed to Express and Secrete Human Growth Hormone (hGH) Under the Control of the AP Promoter and Signal Sequence.

This construction is shown in Figs. 4a-4c. A DNA fragment containing a portion of the AP gene was isolated from the plasmid pHI-1 20 [Inouye, H., et al., J. Bacteriol. 146: 668-675 (1981)]. This was done using a series of steps to introduce an EcoRI site 5' to the AP gene and promoter sequence. The plasmid 20 was digested with HpaI and then ligated to a linker molecule containing two EcoRI sites in tandem. After heat inactivation of the ligase enzyme the DNA was digested with EcoRI and a 1200 bp fragment isolated. This DNA fragment was then treated with HpaII and a 464 bp fragment 21 isolated. A plasmid 22 containing cDNA prepared from human growth hormone mRNA was prepared as described by Martial et al., "Science" 205: 602-606 (1979) and Roskem et al., "Nucleic Acids Res." 7: 305-320 (1979) (see also European Publication No. 127305). Plasmid 22 was digested with HpaII and a 461 bp fragment was isolated. The 461 bp fragment was further digested with PstI and a 200 bp fragment 23 containing part of the hGH gene then

isolated. DNA fragments 21 and 23 were ligated to a 3609bp DNA fragment isolated from pBR322 that had been previously digested with EcoRI and PstI. This DNA ligation mixture was used to transform E. coli 294 to tetracycline resistance. A plasmid 24, designated pcHGHpps, was recovered from a transformant colony.

In plasmid 24 the gene encoding the AP promoter and signal sequence in pcHGHpps was linked to hGH in the same reading frame. However a number of extraneous nucleotides were present between the signal sequence and the beginning of the mature hGH gene. This extraneous nucleotide sequence was deleted by mutagenesis. pcHGHpps was digested with EcoRI and PstI and the 663 bp fragment 25 isolated. Fragment 25 was introduced into M13mp9 previously digested with PstI and EcoRI. This was ligated and used to transform E. coli JM101. Clear plaques were selected and a derivative phage 26, M13mp9-cHGHpps, was identified and isolated. Phage 26 was annealed to the synthetic oligonucleotide primer 5'PCTGTGACAAAAGCCTTCCCAACCATTCC-OH3'. The first 14 nucleotides correspond to the sequence of the 3' end of the AP signal peptide and the last 14 correspond to the 5' end of the mature hGH coding sequence. The site-specific deletion mutagenesis was performed as previously described in Example 3 (see also J. Adelman et al. op cit.). Plaques containing the desired deletion were detected by Southern analysis with the 5'-32P-labeled oligonucleotide primer of this Example. Without enrichment for the desired genotype, nine percent of the plaques screened hybridized to the labelled primer. One of the positives, M13mp9-AP-1, 27, was determined by the dideoxy chain termination nucleotide sequencing method to have the expected sequence. The partial hGH gene, now correctly fused to the AP promoter and signal gene, was introduced into pHGH207, 28 (H. de Boer et al., op cit). This was accomplished by digesting pHGH207 with PstI and NdeI, and then isolating the 2750 bp fragment. Another sample of pHGH207 was digested with NdeI and EcoRI and a 2064 bp fragment isolated. The M13mp9-AP-1 phage was digested by EcoRI and PstI, and the 602bp AP hGH partial gene 29 was isolated. The 2750 and 2064 bp fragments were ligated in a three-part ligation with fragment 29, and the ligation mixture then used to transform E. coli 294 to ampicillin resistance. pAP-1 was isolated from a resistant colony and characterized by restriction enzyme mapping and nucleotide sequence analysis. N.B. This is not an embodiment of the invention.

Example 6

Construction of a Plasmid (pAP-STII-hGH) to Express and Secrete hGH Under the Control of the AP Promoter

ptrp-STII-hGH (from Example 3) was digested with HpaI and EcoRI and the vector fragment 31 isolated. A 420 bp AP promoter fragment 32 was isolated from the plasmid pAP-1 after digestion with EcoRI and partial digestion with RsaI. Fragments 31 and 32 were ligated and used to transform E. coli 294 to ampicillin resistance. The plasmid pAP-STII-hGH was isolated and characterized by restriction enzyme mapping and nucleotide sequence analysis. The nucleotide sequence and translated amino acid sequence of the AP-STII-hGH construction is shown in figure 5.

Example 7

Recovery of hGH from E. coli Containing the Plasmid pAP-STII-hGH.

E. coli W3110 and 294 were transformed respectively with pAP-STII-hGH or pAP-1 and cultured as described in Example 4 except that the medium used was phosphate depleted. The amounts synthesized and the distribution of processed and unprocessed hGH were determined as described in Example 4. The results are described in Table 1. In small volumes ptrp-STII-hGH produces better results, as can be seen from Table 1, but in 10 liter culture volumes the preferred embodiment is the plasmid pAP-STII-hGH since AP promoted cells can grow to higher densities than trp promoted organisms.

Example 8

Large Volume Fermentation and hGH Recovery

Eight hours prior to the start of a 10 liter fermentation a 500 ml inoculum culture is grown up. A transformant of E. coli W3110 (tonA, phoA, phoT) (Example 9) containing pAP-STII-hGH is inoculated into a sterile 2 liter flask containing 500 ml of LB medium and tetracycline (0.5 μg/ml). The culture is incubated in a rotary shaker at 37°C for eight hours. A sterile 10 liter fermentation medium is prepared, containing the following ingredients: 26g K_2HPO_4, 13g NaH_2PO_4 $2H_2O$, 15g KCl, 50g $(NH_4)_2SO_4$, 10g Na_3 citrate, 50 ml of

50 percent glucose, 1000 ml of 10 percent NZ-amine YT, 100 ml of 1M $MgSO_4$, 5 ml of 2.7 percent $FeCl_3$, 5 ml of trace metals, 1 ml of 5 mg/ml tetracycline, 5 ml of antifoaming agent, and 6.5 liters of H_2O. The starting pH of the medium is titrated to 7.5 by adding H_2SO_4, and the run is begun by seeding the 500 ml inoculum culture into the 10 liter fermenter. Throughout the run the temperature is maintained at 37°C and the culture agitated under aeration. From the outset, the cells are fed glucose (50 percent) at a flow rate of 0.5 ml/min. When the OD 550 is in the range 10-25 the glucose feed rate is manually adjusted to keep the pH at 7.5 and the residual glucose level ≤ 1/4 percent. When the OD 550 reaches 25, the glucose feed rate is manually adjusted to drive the dO_2 level to 30 percent and thereafter, the glucose feed rate is periodically adjusted to maintain the dO_2 level at 30 percent. Thirty-six hours after the start of fermentation the cells are killed and harvested.

The glucose feed and aeration are turned off but the agitation rate of 650 rpm is maintained. 1-butanol is added immediately to the fermenter to yield a final concentration of 1.5 percent and steam is immediately injected into the fermenter jacket so that the temperature in the tank rises rapidly to 50°C. When the temperature reaches 50°C, it is held at this temperature for 10 minutes. Then the fermenter is rapidly cooled below 20°C and the cellular contents of the fermenter are harvested by centrifugation. The cell paste is first frozen at -20°C and then transferred to a -80°C freezer.

The cell paste, frozen at -80°C, is thawed overnight at 4°C and all subsequent steps are performed at 4°C. The paste is mixed in 4 volumes of 10mM Tris-HCl,pH=8.0 and suspended in an Ultra-Turrex homogenizer for 30 seconds. The suspension is stirred for 30 minutes and then the cells are removed by centrifugation at 12,000 x g for 30 minutes. The periplasmic proteins contain in the supernatant mature hGH at between 0.5 and 1 gram/liter/100 OD_{550} with about 95 percent of the total hGH (as estimated from immunoblots with peroxidase-coupled anti-hGH) in processed form in the periplasm. About 50-60 percent of the total cellular hGH is recovered in the supernatant. The supernatant contains about 20 percent hGH by weight of protein.

Example 9

Construction of a Host Organism W3110 tonA, phoA, phoT

The host organism for the fermentation was constructed in several steps using standard techniques involving transductions with phage derived from P1 (J. Miller, Experiments in Molecular Genetics). The phoT and phoA mutations were sequentially cotransduced from E. coli into strain W3110 tonA with the aid of genetically linked antibiotic-resistance transposons. The presence of the phoT mutation, which was introduced first, was recognized since these transductants form blue colonies on high phosphate, phosphochromogen-containing plates (5-bromo-4-chloro-3-indoylphosphate).The introduction of the phoA mutation was recognized as transductants which form white colonies on low phosphate, phosphochromogen plates. This phoT mutant, or a phoR mutant constructed in similar fashion, when transformed with pAP-STII-hGH, secretes over 90 percent of the total hGH into the periplasmic space over the course of the fermentation. In contrast, a non-constitutive bacterium such as W3110 only secretes about 50 percent of the hGH into the periplasm and total expression levels are somewhat lower in some cases. The presence or absence of the phoA mutant makes little or no difference in the yield of heterologous protein. However, since phoA mutants do not secrete AP, it is not necessary to separate AP in the course of purifying periplasmic hGH from phoA mutants.

Claims

1. A hybrid DNA sequence encoding a protein having at least the amino terminal sequence of mature hGH operably linked to a DNA sequence encoding an enterotoxin signal.

2. A hybrid DNA sequence according to claim 1 wherein the signal is the STII signal.

3. A replicable vector containing, in 5' to 3' order, a promoter, a Shine-Dalgarno sequence, a hybrid DNA sequence according to claim 1 or claim 2, and a termination region comprising a stop codon; said promoter controlling transcription of the hybrid DNA sequence.

4. A method comprising;
   (a) constructing a vector comprising a DNA sequence encoding a protein having at least the amino terminal sequence of mature hGH operably ligated to a DNA sequence encoding an enterotoxin

signal, the DNA being operably linked to the STII Shine-Dalgarno sequence;
(b) transforming a host cell capable of processing the hybrid and secreting the mature eukaryotic protein with the vector;
(c) culturing the transformed host cell; and
(d) recovering the mature protein which comprises at least the amino terminal sequence of hGH from the periplasm of the cell.

5. A prokaryotic cell culture comprising;
(a) a mature eukaryotic protein and
(b) a hybrid DNA encoding a direct fusion protein of an enterotoxin signal directly fused at its carboxyl terminus to the amino terminus of the mature eukaryotic protein; and wherein the mature protein is located in the periplasm of the cell.

6. A prokaryotic cell culture comprising;
(a) mature hGH and
(b) a hybrid DNA encoding a direct fusion protein of an enterotoxin signal directly fused at its carboxyl terminus to the amino terminus of mature hGH; and wherein the mature protein is located in the periplasm of the cell.

7. A cell culture according to claim 5 or 6 wherein greater than about 25% of the total weight of mature and fusion protein is located in the cell periplasm.

8. The culture of claim 5, 6 or 7 wherein the total weight of mature and fusion protein is greater than about 2mg/litre of culture on a culture optical density basis of 1 at 550 nm and greater than 50 percent of the mature protein by weight is present in the periplasm.

9. The culture of claim 8 wherein said total weight is greater than 4mg/litre.

10. The culture of claim 8 or 9 wherein greater than 80% of the mature protein by weight is present in the periplasm.

11. A protein comprising an enterotoxin signal directly fused at its carboxyl terminus to the amino terminus of a mature eukaryotic protein.

12. A method according to claim 4 wherein said step of recovering the mature protein from the periplasmic space comprises:
(a) killing the cell;
(b) freezing the cell;
(c) thawing the cell; and
(d) separating the periplasmic proteins, including the eukaryotic protein, from the remainder of the cells.

13. The method of claim 12 wherein the cells are killed by
(a) contacting the cell with an alkanol which is not lytic for cell membranes, and;
(b) heating the cell.

14. A method of claim 13 wherein the cell is cultured in a phosphate-limiting culture medium prior to freezing the cell.

15. A method for the recovery of mature hGH from a bacterial cell comprising carrying out a method according to claim 6 wherein said DNA sequence encodes the full sequence of mature hGH; and wherein said recovery step (d) comprises:
(e) killing the cell;
(f) freezing the cell; and
(g) separating periplasmic protein from the remainder of the cell.

Revendications

1. Séquence d'ADN hybride codant pour une protéine ayant au moins la séquence amino-terminale de la hGH mature liée de manière fonctionnelle à une séquence d'ADN codant pour un signal d'entérotoxine.

2. Séquence d'ADN hybride suivant la revendication 1, dans laquelle le signal est le signal STII.

3. Vecteur d'ADN réplicable contenant, dans le sens 5' à 3', un promoteur, une séquence Shine-Dalgarno, une séquence d'ADN hybride suivant la revendication 1 ou la revendication 2, et une région de terminaison comprenant un codon d'arrêt ; ledit promoteur régulant la transcription de la séquence d'ADN hybride.

4. Procédé consistant :
   (a) à construire un vecteur comprenant une séquence d'ADN codant pour une protéine ayant au moins la séquence amino-terminale de la hGH mature réunie par ligation fonctionnelle à une séquence d'ADN codant pour un signal d'entérotoxine, l'ADN étant lié de manière fonctionnelle à la séquence Shine-Dalgarno de STII ;
   (b) à transformer une cellule-hôte capable d'effectuer la maturation de l'hybride et de sécréter la protéine eucaryotique mature avec le vecteur ;
   (c) à cultiver la cellule-hôte transformée ; et
   (d) à recueillir la protéine mature qui comprend au moins la séquence amino-terminale de la hGH à partir du périplasme de la cellule.

5. Culture de cellules procaryotiques, comprenant :
   (a) une protéine eucaryotique mature, et
   (b) un ADN hybride codant pour une protéine de fusion directe d'un signal d'entérotoxine fusionné directement à son extrémité carboxyle à l'extrémité amino de la protéine eucaryotique mature ; la protéine mature étant présente dans le périplasme de la cellule.

6. Culture de cellules procaryotiques comprenant :
   (a) la hGH mature, et
   (b) un ADN hybride codant pour une protéine de fusion directe d'un signal d'entérotoxine fusionné directement à son extrémité carboxyle à l'extrémité amino de la hGH mature ; la protéine mature étant présente dans le périplasme de la cellule.

7. Culture de cellules suivant la revendication 5 ou 6, dans laquelle une quantité supérieure à environ 25% du poids total de la protéine mature et de fusion est présente dans le périplasme de la cellule.

8. Culture suivant la revendication 5, 6 ou 7, dans laquelle le poids total de la protéine mature et de fusion est supérieur à environ 2 mg/litre de culture, sur la base d'une densité optique de la culture de 1 à 550 nm, et plus de 50% de la protéine mature, en poids, sont présents dans le périplasme.

9. Culture suivant la revendication 8, dans laquelle le poids total est supérieur à 4 mg/litre.

10. Culture suivant la revendication 8 ou 9, dans laquelle plus de 80% de la protéine mature, en poids, sont présents dans le périplasme.

11. Protéine comprenant un signal d'entérotoxine fusionné directement à son extrémité carboxyle à l'extrémité amino d'une protéine eucaryotique mature.

12. Procédé suivant la revendication 4, dans lequel l'étape de séparation de la protéine mature de l'espace périplasmique consiste :
   (a) à tuer la cellule ;
   (b) à congeler la cellule ;
   (c) à décongeler la cellule ; et
   (d) à séparer les protéines périplasmiques, comprenant la protéine eucaryotique, du reste des cellules.

13. Procédé suivant la revendication 12, dans lequel les cellules sont tuées
   (a) par mise en contact de la cellule avec un alcanol qui ne possède pas d'action de lyse sur les

membranes cellulaires, et
(b) par chauffage de la cellule.

14. Procédé suivant la revendication 13, dans lequel la cellule est cultivée dans un milieu de culture limitant en phosphate avant congélation de la cellule.

15. Procédé pour séparer de la hGH mature d'une cellule bactérienne, consistant à mettre en oeuvre un procédé suivant la revendication 6, dans lequel la séquence d'ADN code pour la séquence totale de la hGH mature ; et dans lequel l'étape de séparation (d) consiste :
(e) à tuer la cellule ;
(f) à congeler la cellule ; et
(g) à séparer la protéine périplasmique du reste de la cellule.

Patentansprüche

1. Hybrid-DNA-Sequenz, die für ein Protein kodiert, bei dem zumindest die Aminoterminalsequenz von reifem hGH mit einer DNA-Sequenz operabel verbunden ist, die für ein Enterotoxinsignal kodiert.

2. Hybrid-DNA-Sequenz nach Anspruch 1, worin das Signal das STII-Signal ist.

3. Replizierbarer Vektor, der in 5' bis 3' Reihenfolge einen Promotor, eine Shine-Dalgarno-Sequenz, eine Hybrid-DNA-Sequenz nach Anspruch 1 oder 2 und einen Terminationsbereich aufweist, der ein Stopcodon umfaßt; wobei der genannte Promotor die Transkription der Hybrid-DNA-Sequenz steuert.

4. Verfahren, umfassend:
(a) das Konstruieren eines Vektors, der eine DNA-Sequenz umfaßt, die für ein Protein kodiert, bei dem zumindest die Aminoterminalsequenz von reifem hGH mit einer DNA-Sequenz operabel ligiert ist, die für ein Enterotoxinsignal kodiert, wobei die DNA operabel mit der STII-Shine-Dalgarnosequenz verbunden ist;
(b) das Transformieren einer Wirtszelle, die fähig ist, das Hybrid zu verarbeiten und das reife eukaryotische Protein mit dem Vektor auszuscheiden;
(c) das Kultivieren der transformierten Wirtszelle; und
(d) das Wiedergewinnen des reifen Proteins, das zumindest die aminoterminale Sequenz von hGH umfaßt, aus dem Periplasma der Zelle.

5. Prokaryotische Zellkultur, umfassend:
(a) ein reifes eukaryotisches Protein und
(b) eine Hybrid-DNA, die für ein direktes Fusionsprotein eines Enterotoxinsignals kodiert, das an seinem Carboxylterminus direkt mit dem Aminoterminus des reifen eukaryotischen Proteins verschmolzen ist; und worin das reife Protein sich im Periplasma der Zelle befindet.

6. Prokaryotische Zellkultur, umfassend:
(a) reifes hGH und
(b) eine Hybrid-DNA, die für ein direktes Fusionsprotein eines Enterotoxinsignals kodiert, das an seinem Carboxylterminus direkt mit dem Aminoterminus von reifem hGH verschmolzen ist; und worin das reife Protein sich im Periplasma der Zelle befindet.

7. Zellkultur nach Anspruch 5 oder 6, worin sich mehr als etwa 25% des Gesamtgewichts an reifem und Fusionsprotein im Zellperiplasma befinden.

8. Kultur nach einem der Ansprüche 5, 6 oder 7 , worin das Gesamtgewicht an reifem und Fusionsprotein größer als etwa 2mg/Liter Kultur auf einer optischen Dichtebasis der Kultur von 1 bei 550 nm ist, und mehr als 50 Gew.-% des reifen Proteins im Periplasma vorhanden sind.

9. Kultur nach Anspruch 8, worin das genannte Gesamtgewicht größer als 4 mg/Liter ist.

10. Kultur nach Anspruch 8 oder 9, worin mehr als 80 Gew.-% des reifen Proteins im Periplasma vorhanden sind.

11. Protein, das ein Enterotoxinsignal umfaßt, das an seinem Carboxylterminus direkt mit dem Aminoterminus eines reifen eukaryotischen Proteins verschmolzen ist.

12. Verfahren nach Anspruch 4, worin der genannte Schritt des Wiedergewinnens des reifen Proteins vom periplasmischen Raum umfaßt:
(a) das Abtöten der Zelle;
(b) das Einfrieren der Zelle;
(c) das Auftauen der Zelle; und
(d) das Abtrennen des periplasmischen Proteins, einschließlich des eukaryotischen Proteins, vom Rest der Zellen.

13. Verfahren nach Anspruch 12, worin die Zellen abgetötet werden durch
(a) In-Kontakt-bringen der Zelle mit einem Alkanol, das nicht lytisch für Zellmembranen ist, und
(b) Erwärmen der Zelle.

14. Verfahren nach Anspruch 13, worin die Zelle vor dem Einfrieren der Zelle in einem phosphatbeschränkenden Kulturmedium kultiviert wird.

15. Verfahren zur Wiedergewinnung von reifem hGH aus einer bakteriellen Zelle, welches die Durchführung eines Verfahrens nach Anspruch 6 umfaßt, worin die genannte DNA-Sequenz für die volle Sequenz von reifem hGH kodiert; und worin der genannte Wiedergewinnungsschritt
(d) umfaßt:
(e) das Abtüten der Zelle
(f) das Einfrieren der Zelle; und
(g) das Abtrennen des periplasmischen Proteins vom Rest der Zelle.

THE ST II GENE

```
TAAATACCTACAACGGGTGATTGACACTACACTCATTAACTATACTGCAAGTAGCATTAAAAATCTTAATAAAGGAGAGC
1                  20                  40                  60                  80

   -35                      -10                                        MnlI   S.D.
TTCGTCACATTTTTTTGACTTGACTCATATAAAAGCCCACTGGTATAAGTTTTATTGCTTATAGCAATAAGGTTGAGGTG
         .          100                 120                 140                 160

                   -20                                  -10
       met lys lys asn ile ala phe leu leu ala ser met phe val phe ser ile ala
ATTTT  ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT
                        180                 200

                -1 cleavage site                    10
thr asn ala tyr ala SER THR GLN SER ASN LYS LYS ASP LEU CYS GLU HIS TYR ARG GLN
ACA AAT GCC TAT GCA TCT ACA CAA TCA AAT AAA AAA GAT CTG TGT GAA CAT TAT AGA CAA
220                 240                 BglII 260

                20                                  30
ILE ALA LYS GLU SER CYS LYS LYS GLY PHE LEU GLY VAL ARG ASP GLY THR ALA GLY ALA
ATA GCC AAG GAA AGT TGT AAA AAA GGT TTT TTA GGG GTT AGA GAT GGT ACT GCT GGA GCA
280                 300                 320     RsaI

                40
CYS PHE GLY ALA GLN ILE MET VAL ALA ALA LYS GLY CYS OC
TGC TTT GGC GCC CAA ATA ATG GTT GCA GCA AAA GGA TGC TAA TATATTTATCAATAGCATTCAGCA
340                 360                 380                 400

CCATATACACAAAAATAATTTTTCATAAAAAGAACTCTATAAAATAAATATTTTTTGTGACAATGTCCTAACGCAAGACG
          420                 440                 460                 480

GACATTGTCCATTTCTCACTGCAGGTAAATGATCTGTAAATAGTC
          500  PstI           520
```

Fig.1.

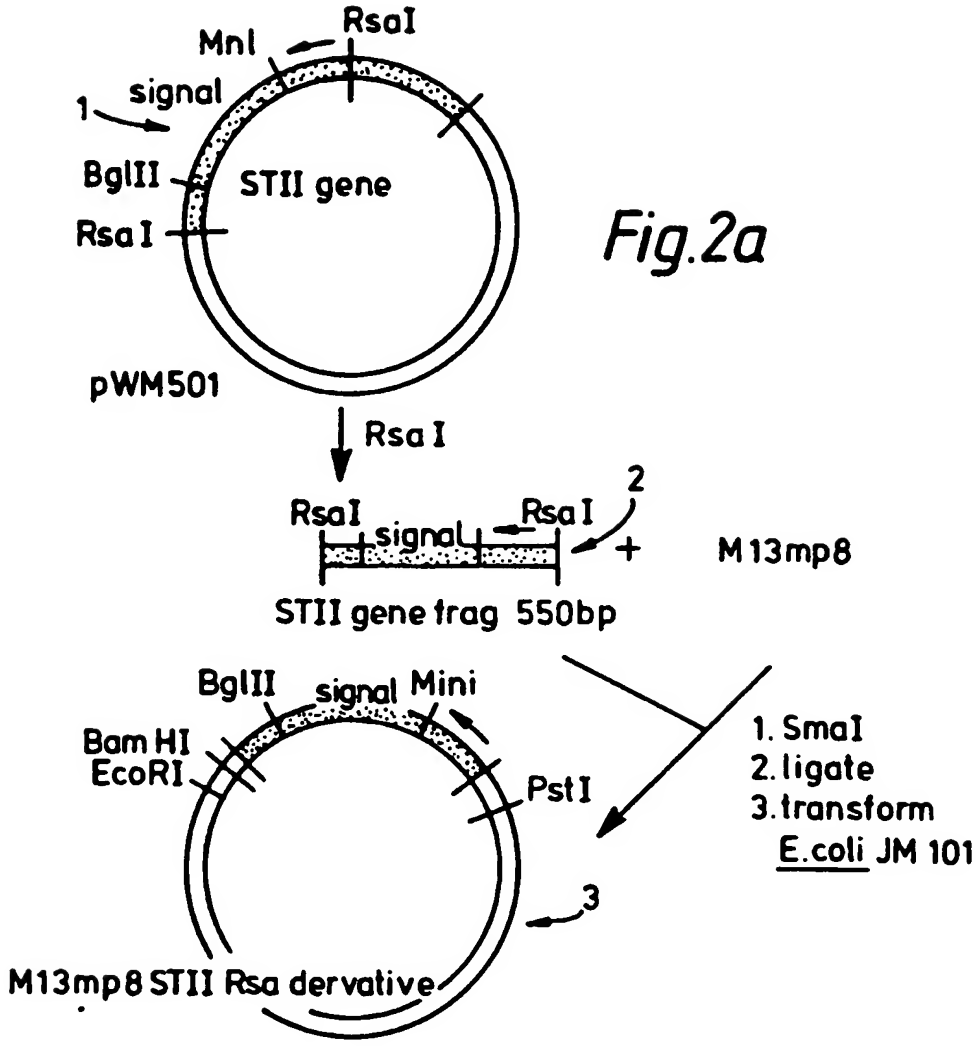

MnI
RsaI
signal
1
BglII
STII gene
Rsa I
pWM501
Rsa I
RsaI
signal
RsaI
2
+
M13mp8
STII gene frag 550bp
BglII
signal
MinI
Bam HI
EcoRI
PstI
3
M13mp8 STII Rsa dervative
1. SmaI
2. ligate
3. transform
E.coli JM 101

Fig.2a

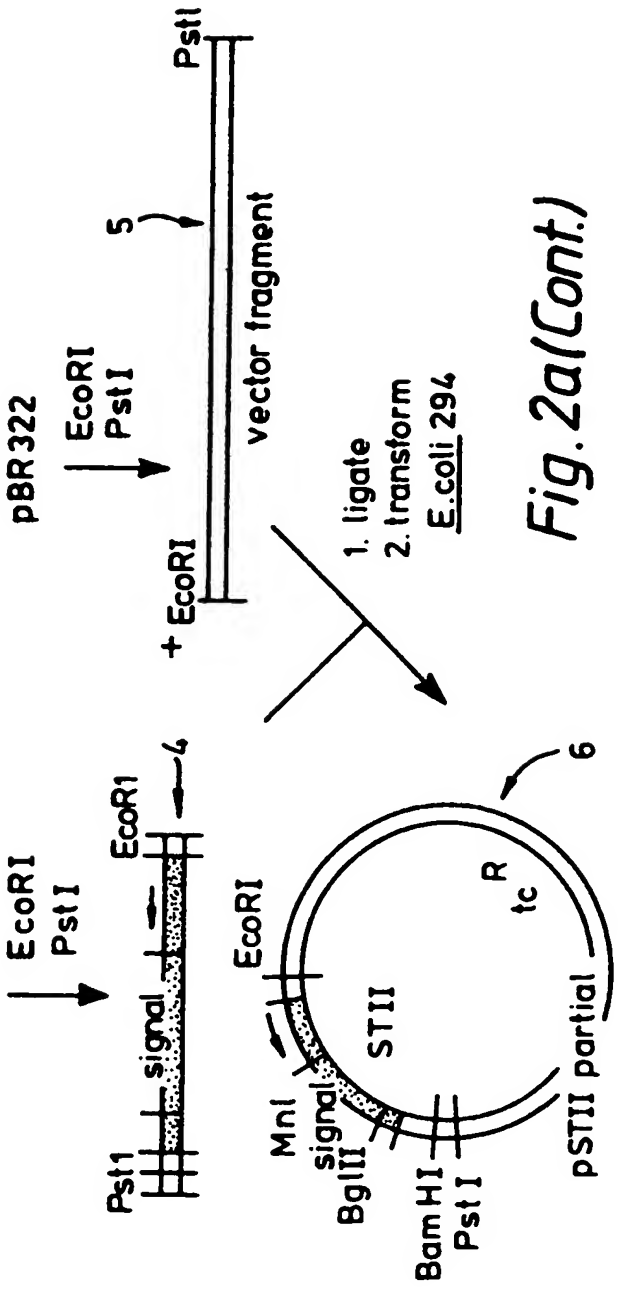

pBR322
EcoRI
PstI
+ EcoRI
5
PstI
vector fragment
1. ligate
2. transform
E.coli 294
EcoRI
PstI
Pst1
signal
EcoR1
4
EcoRI
MnI
signal
BglII
BamHI
PstI
STII
R
tc
pSTII partial
6

Fig. 2a(Cont.)

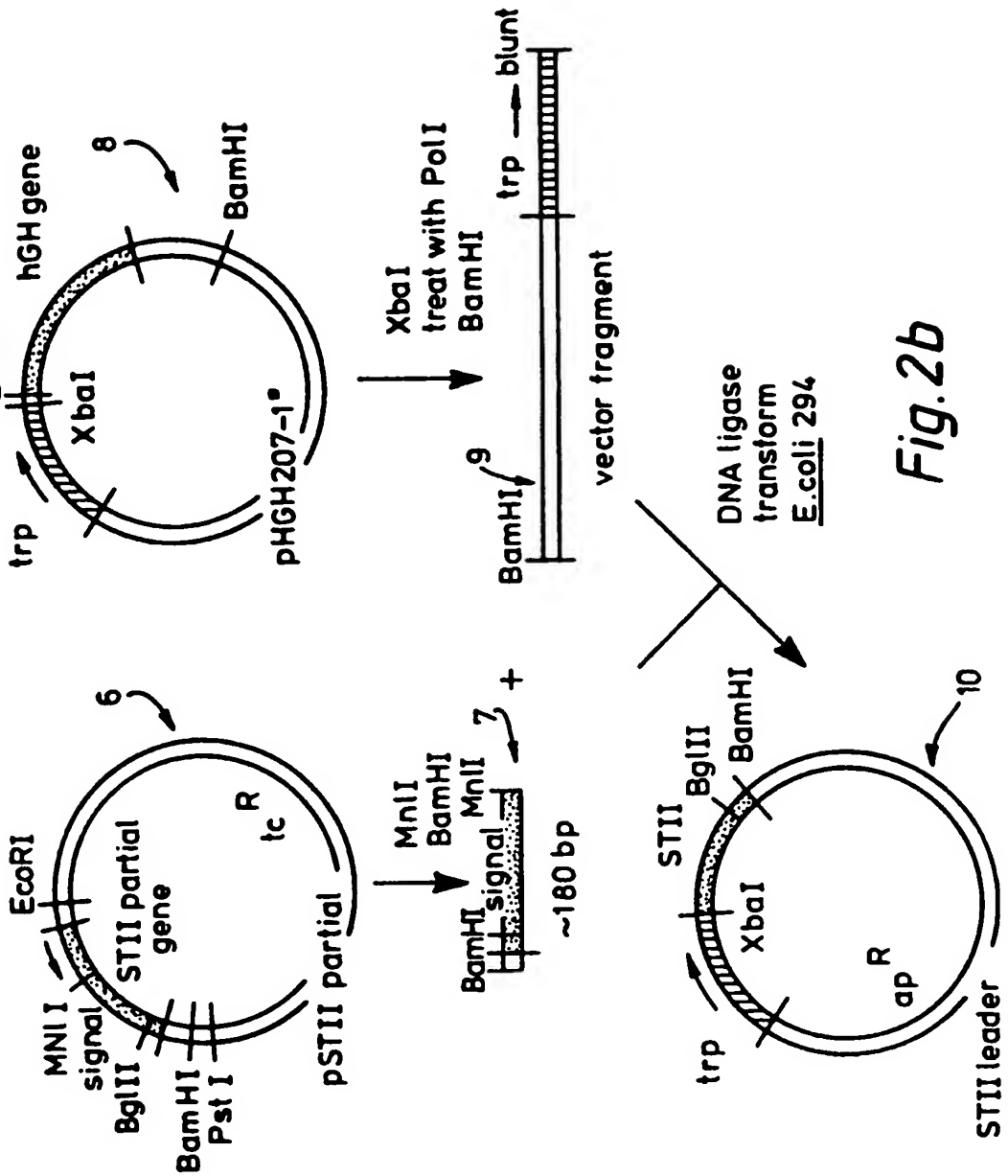
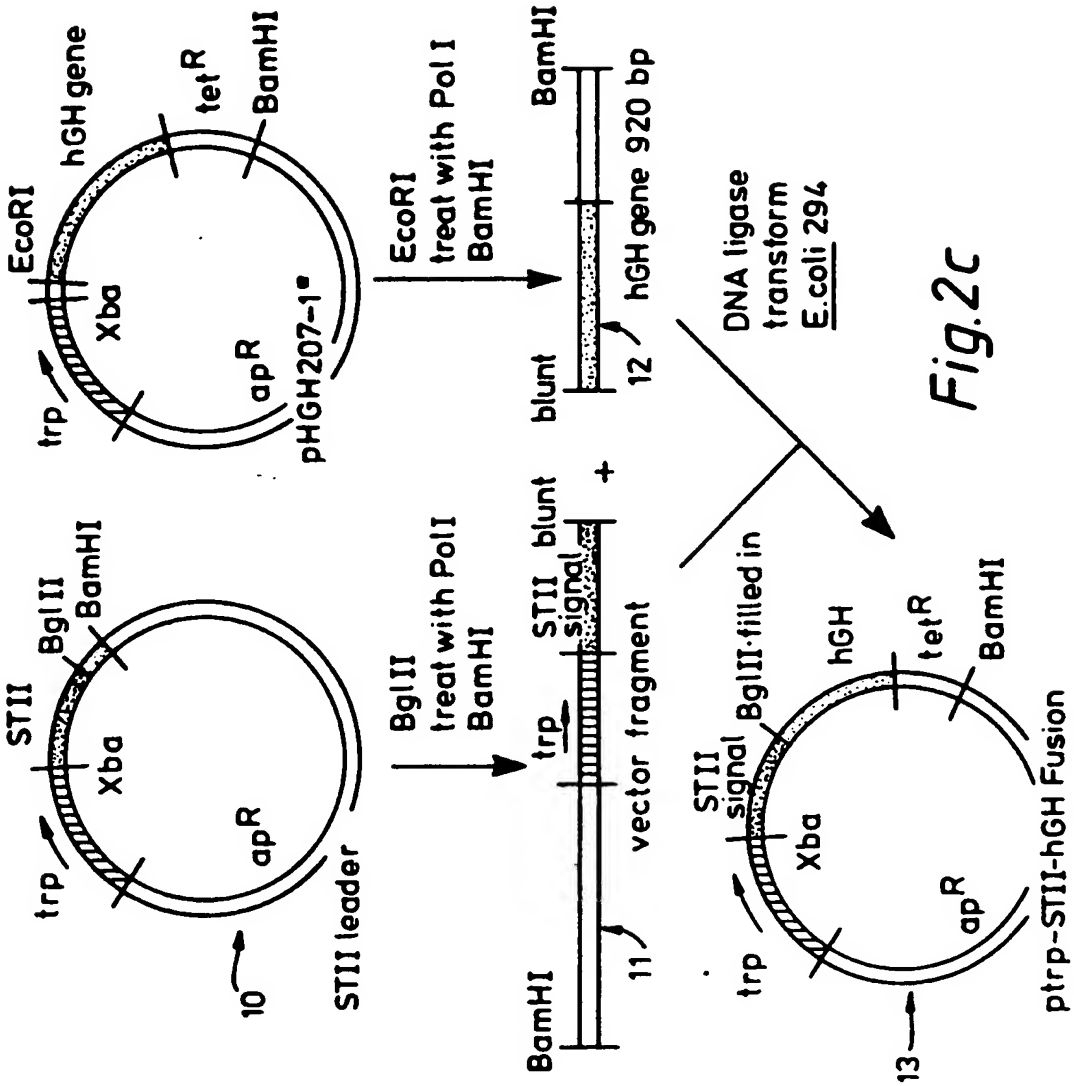

EcoRI
hGH gene
8
BamHI
XbaI
trp
pHGH207-1*
XbaI
treat with PolI
BamHI
trp
blunt
9
BamHI
vector fragment
6
EcoRI
STII partial gene
R
tc
MNl I
signal
BglII
BamHI
Pst I
pSTII partial
MnlI
BamHI
MnlI
7
+
BamHI
signal
~180 bp
DNA ligase
transform
E.coli 294
STII
BglII
BamHI
10
XbaI
trp
R
ap
STII leader

Fig.2b


EcoRI
hGH gene
tet^R
BamHI
Xba
trp
ap^R
pHGH207-1*
EcoRI
treat with Pol I
BamHI
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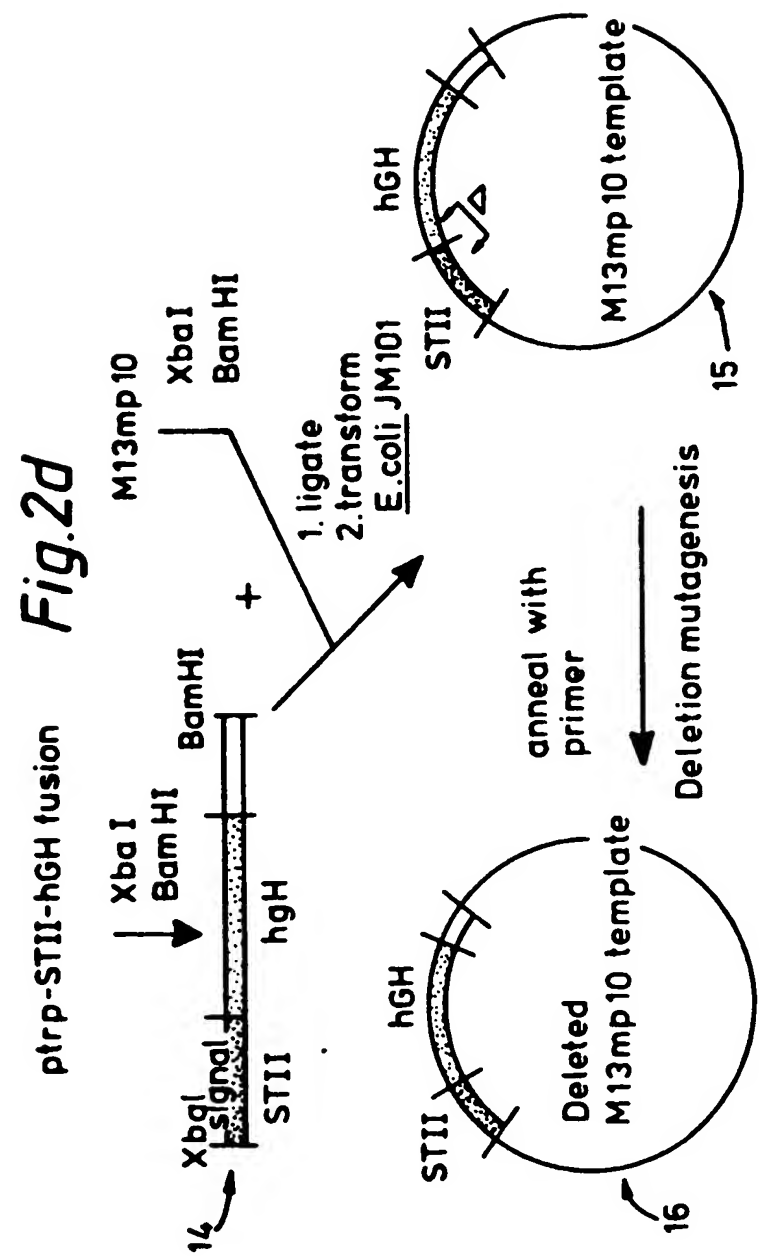
blunt
12
hGH gene 920 bp
DNA ligase
transform
E.coli 294
STII
BglII
BamHI
Xba
trp
ap^R
10
STII leader
BglII
treat with Pol I
BamHI
trp
STII blunt
signal
+
BamHI
11
vector fragment
STII
signal
BglII-filled in
hGH
tet^R
BamHI
Xba
trp
ap^R
13
ptrp-STII-hGH Fusion

Fig.2c

Fig.2d


ptrp-STII-hGH fusion
Xba I
Bam HI
BamHI
hgH
Xbal
Signal
STII
14
M13mp10
Xba I
Bam HI
+
1. ligate
2. transform
E.coli JM101
hGH
STII
M13mp10 template
15
anneal with
primer
Deletion mutagenesis
hGH
STII
Deleted
M13mp10 template
16

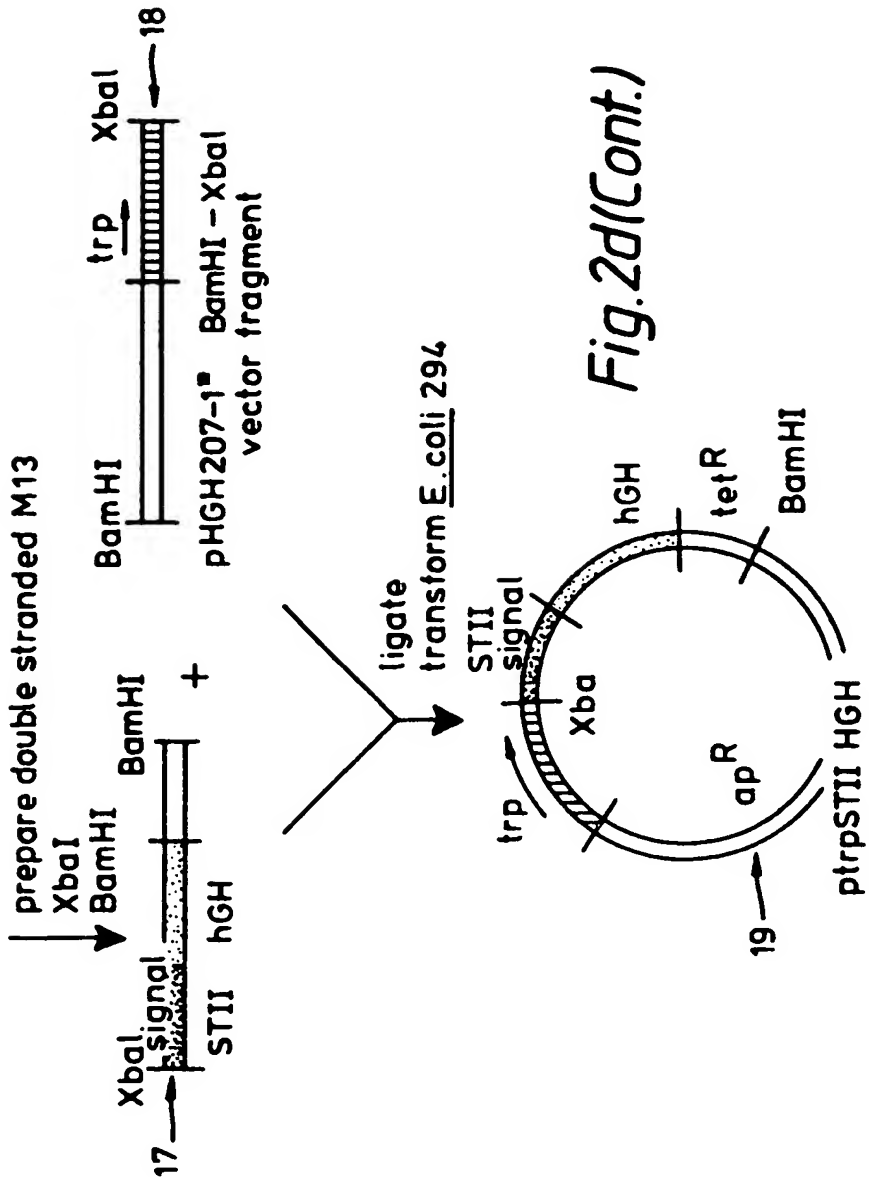

prepare double stranded M13
XbaI
BamHI
XbaI
signal
STII
hGH
17
BamHI
+
BamHI
trp
XbaI
18
pHGH207-1*
BamHI – XbaI
vector fragment
ligate
transform E.coli 294
STII
signal
trp
Xba
hGH
tetR
BamHI
apR
19
ptrpSTII HGH

Fig.2d(Cont.)

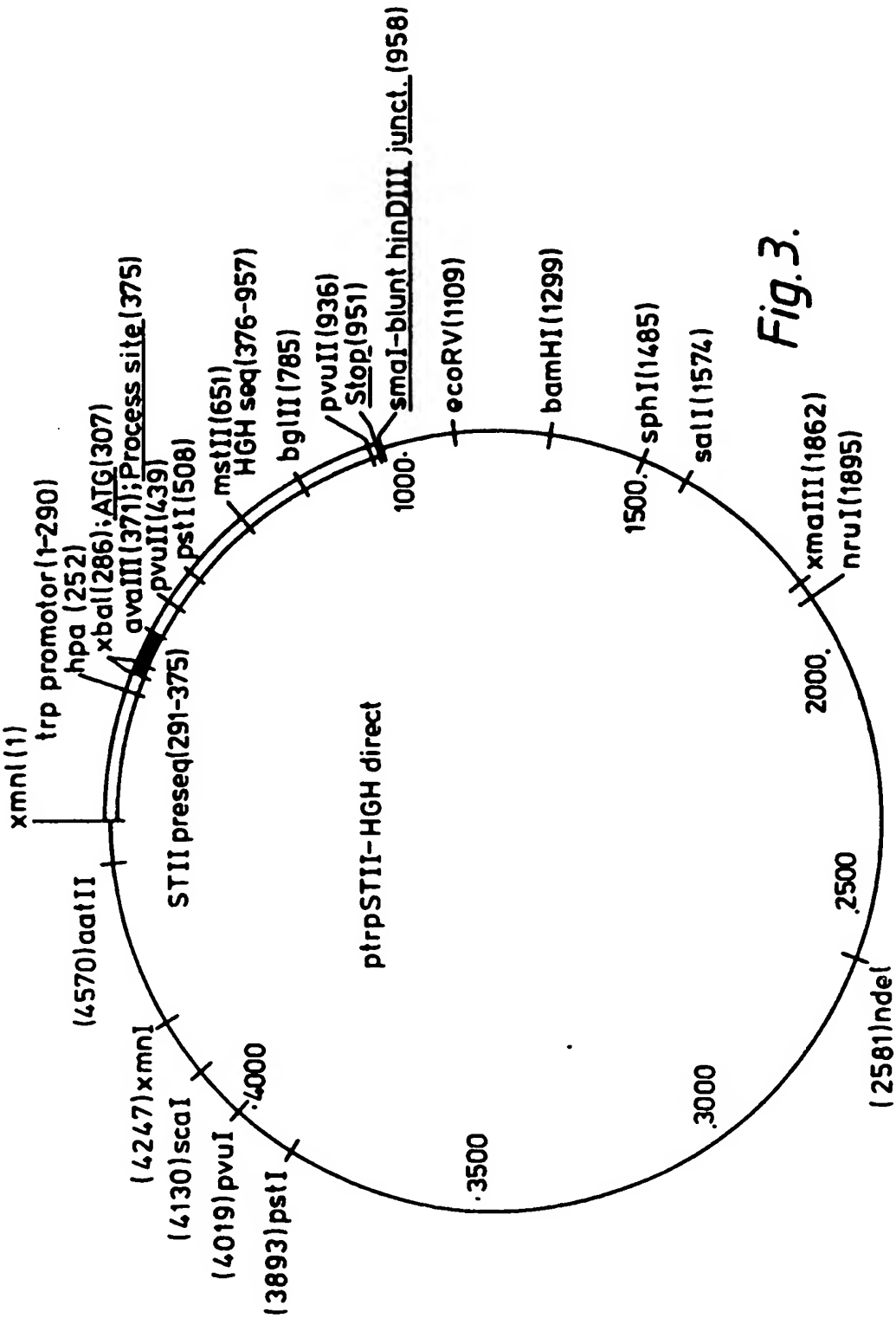
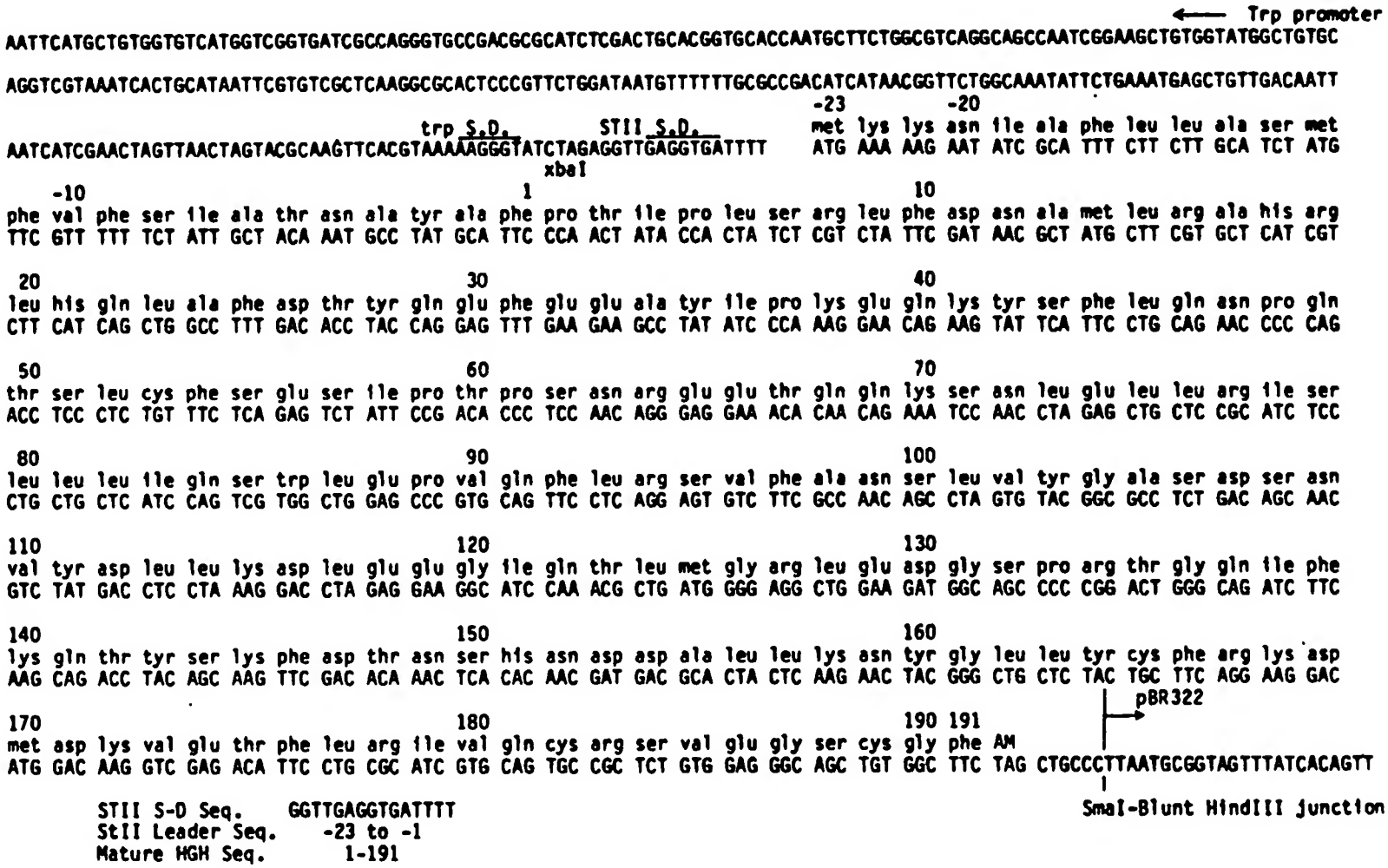

xmnI(1)
trp promotor(1-290)
hpa (252)
xbaI(286);ATG(307)
avaIII(371);Process site(375)
pvuII(439)
pstI(1508)
mstII(651)
HGH seq(376-957)
bglII(785)
pvuII(936)
Stop(951)
smaI-blunt hinDIII junct. (958)
ecoRV(1109)
bamHI(1299)
sphI(1485)
salI(1574)
xmaIII(1862)
nruI(1895)
(2581)ndel
(3893)pstI
(4019)pvuI
(4130)scaI
(4247)xmnI
(4570)aatII
STII preseq(291-375)
ptrpSTII-HGH direct
1000
1500
2000
2500
3000
3500
4000

Fig. 3.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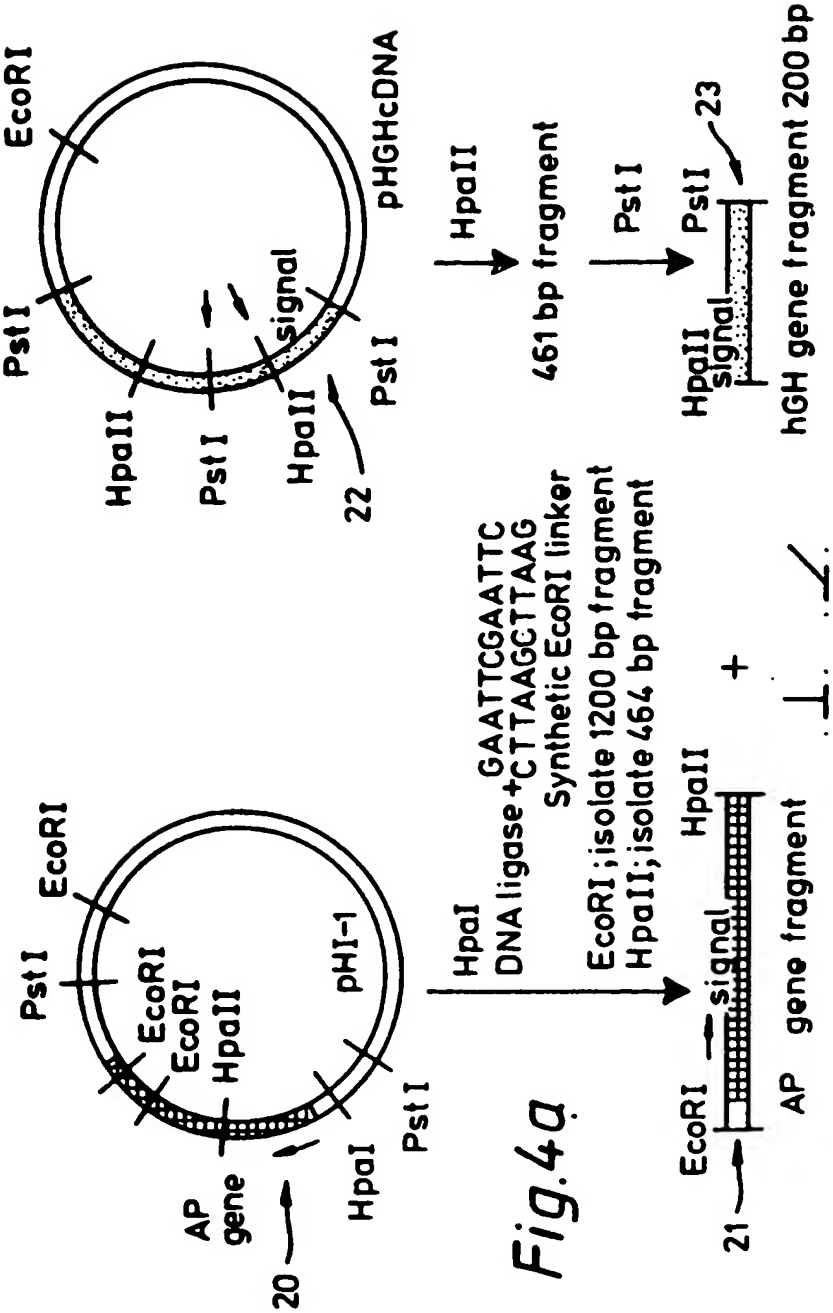
AGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATGAGCTGTTGACAATT
-23 -20
trp S.D. STII S.D.
met lys lys asn ile ala phe leu leu ala ser met
AATCATCGAACTAGTTAACTAGTACGCAAGTTCACGTAAAAAGGGTATCTAGAGGTTGAGGTGATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG
xbaI
-10 1 10
phe val phe ser ile ala thr asn ala tyr ala phe pro thr ile pro leu ser arg leu phe asp asn ala met leu arg ala his arg
TTC GTT TTT TCT ATT GCT ACA AAT GCC TAT GCA TTC CCA ACT ATA CCA CTA TCT CGT CTA TTC GAT AAC GCT ATG CTT CGT GCT CAT CGT
20 30 40
leu his gln leu ala phe asp thr tyr gln glu phe glu glu ala tyr ile pro lys glu gln lys tyr ser phe leu gln asn pro gln
CTT CAT CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT GAA GAA GCC TAT ATC CCA AAG GAA CAG AAG TAT TCA TTC CTG CAG AAC CCC CAG
50 60 70
thr ser leu cys phe ser glu ser ile pro thr pro ser asn arg glu glu thr gln gln lys ser asn leu glu leu leu arg ile ser
ACC TCC CTC TGT TTC TCA GAG TCT ATT CCG ACA CCC TCC AAC AGG GAG GAA ACA CAA CAG AAA TCC AAC CTA GAG CTG CTC CGC ATC TCC
80 90 100
leu leu leu ile gln ser trp leu glu pro val gln phe leu arg ser val phe ala asn ser leu val tyr gly ala ser asp ser asn
CTG CTG CTC ATC CAG TCG TGG CTG GAG CCC GTG CAG TTC CTC AGG AGT GTC TTC GCC AAC AGC CTA GTG TAC GGC GCC TCT GAC AGC AAC
110 120 130
val tyr asp leu leu lys asp leu glu glu gly ile gln thr leu met gly arg leu glu asp gly ser pro arg thr gly gln ile phe
GTC TAT GAC CTC CTA AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG ATG GGG AGG CTG GAA GAT GGC AGC CCC CGG ACT GGG CAG ATC TTC
140 150 160
lys gln thr tyr ser lys phe asp thr asn ser his asn asp asp ala leu leu lys asn tyr gly leu leu tyr cys phe arg lys asp
AAG CAG ACC TAC AGC AAG TTC GAC ACA AAC TCA CAC AAC GAT GAC GCA CTA CTC AAG AAC TAC GGG CTG CTC TAC TGC TTC AGG AAG GAC
pBR322
170 180 190 191
met asp lys val glu thr phe leu arg ile val gln cys arg ser val glu gly ser cys gly phe AM
ATG GAC AAG GTC GAG ACA TTC CTG CGC ATC GTG CAG TGC CGC TCT GTG GAG GGC AGC TGT GGC TTC TAG CTGCCCTTAATGCGGTAGTTTATCACAGTT
SmaI-Blunt HindIII junction
STII S-D Seq. GGTTGAGGTGATTTT
StII Leader Seq. -23 to -1
Mature HGH Seq. 1-191

Fig.3A.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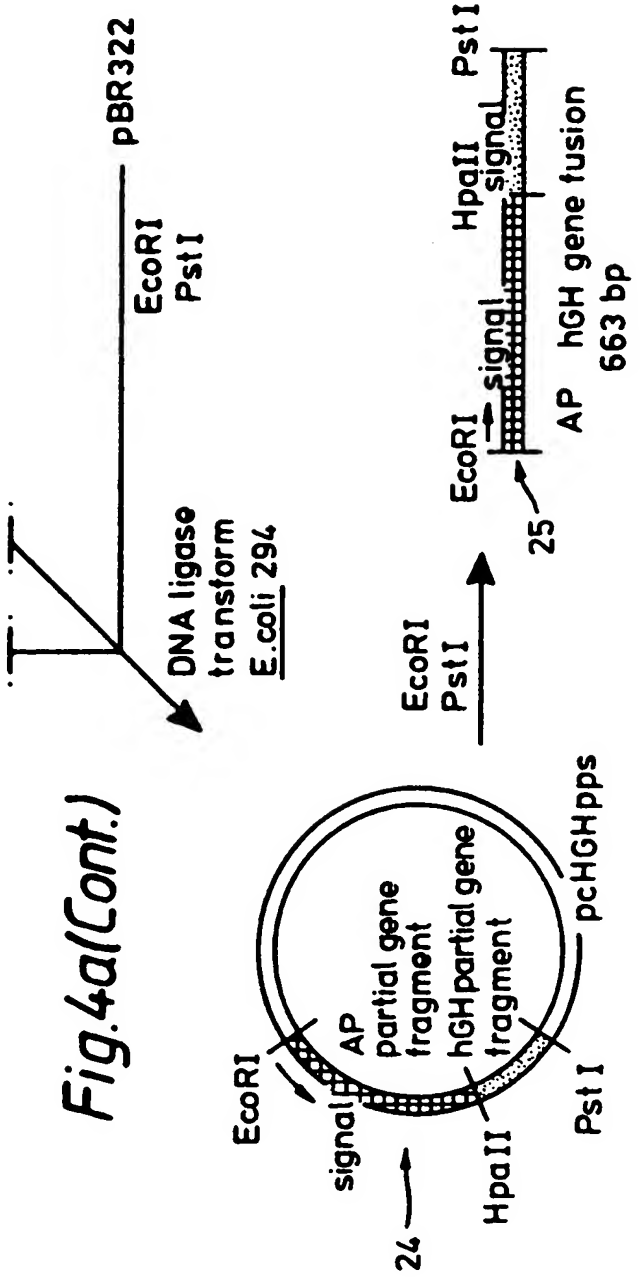
PstI
HpaII
PstI
signal
HpaII
PstI
22
pHGHcDNA
HpaII
461 bp fragment
PstI
23
HpaII
signal
PstI
hGH gene fragment 200 bp
PstI
EcoRI
EcoRI
HpaII
pHI-1
AP
gene
20
HpaI
PstI
HpaI
DNA ligase +
GAATTCGAATTC
CTTAAGCTTAAG
Synthetic EcoRI linker
EcoRI; isolate 1200 bp fragment
HpaII; isolate 464 bp fragment
EcoRI
signal
HpaII
21
AP gene fragment
+

Fig.4a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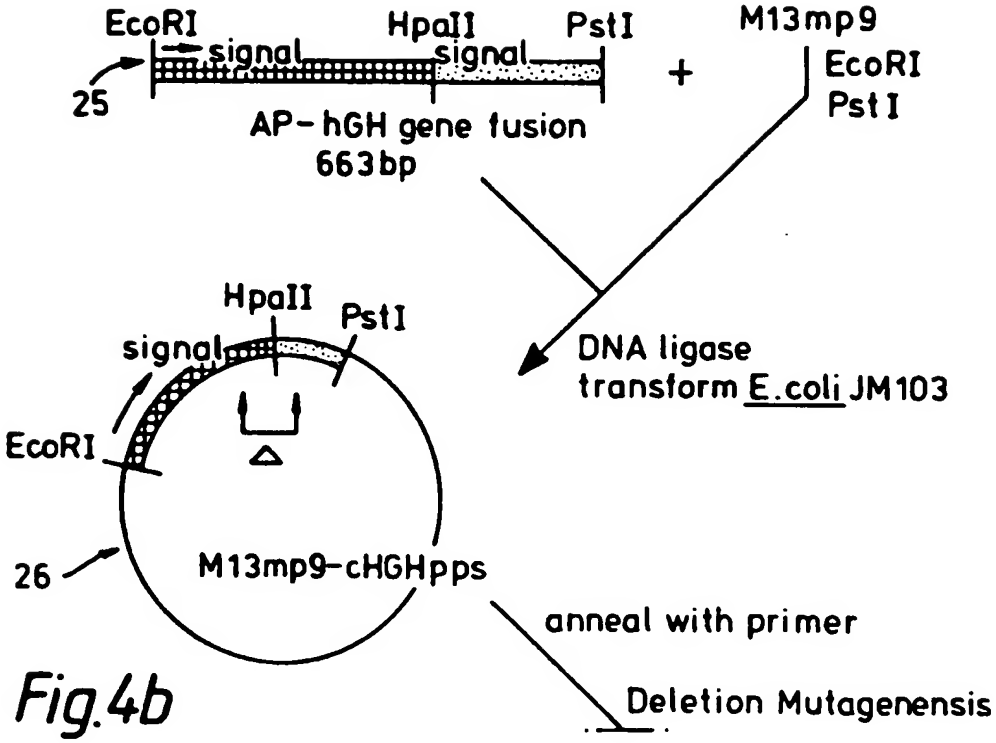
EcoRI
PstI
DNA ligase
transform
E.coli 294
EcoRI
PstI
HpaII
PstI
signal
EcoRI
signal
AP
hGH gene fusion
663 bp
25
EcoRI
signal
AP
partial gene
fragment
hGH partial gene
fragment
pcHGHpps
HpaII
PstI
24

Fig.4a(Cont.)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PstI
signal
signal
25
AP-hGH gene fusion
663bp
+
M13mp9
EcoRI
PstI
DNA ligase
transform E.coli JM103
HpaII
PstI
signal
EcoRI
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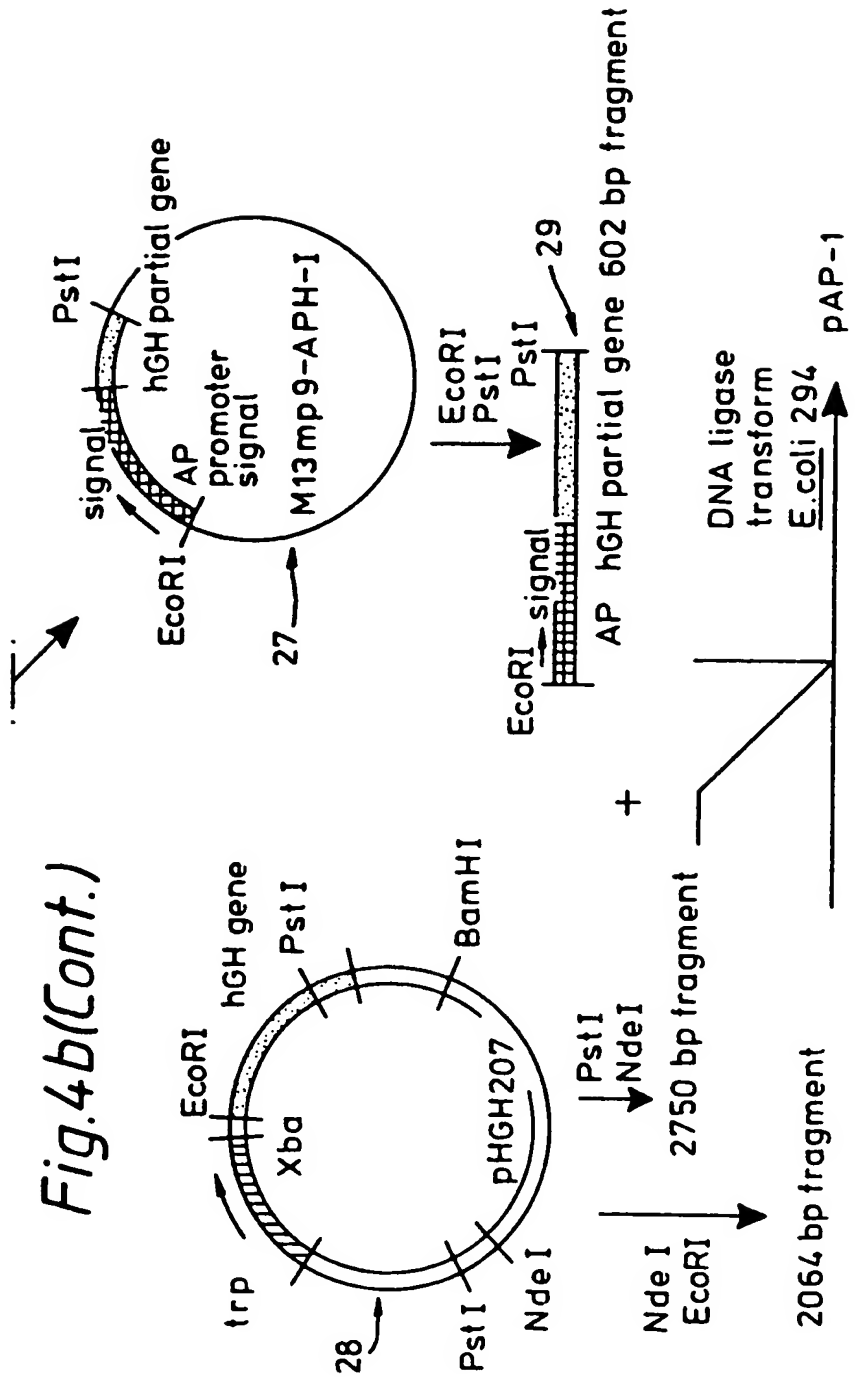
M13mp9-cHGHpps
anneal with primer
Deletion Mutagenensis

Fig.4b

Fig.4b(Cont.)


PstI
hGH partial gene
signal
AP
promoter
signal
M13mp9-APH-I
EcoRI
27
EcoRI
PstI
PstI
29
EcoRI
signal
AP hGH partial gene 602 bp fragment
DNA ligase
transform
E.coli 294
pAP-1
+
EcoRI
hGH gene
PstI
BamHI
Xba
pHGH207
trp
28
PstI
NdeI
NdeI
EcoRI
2750 bp fragment
2064 bp fragment

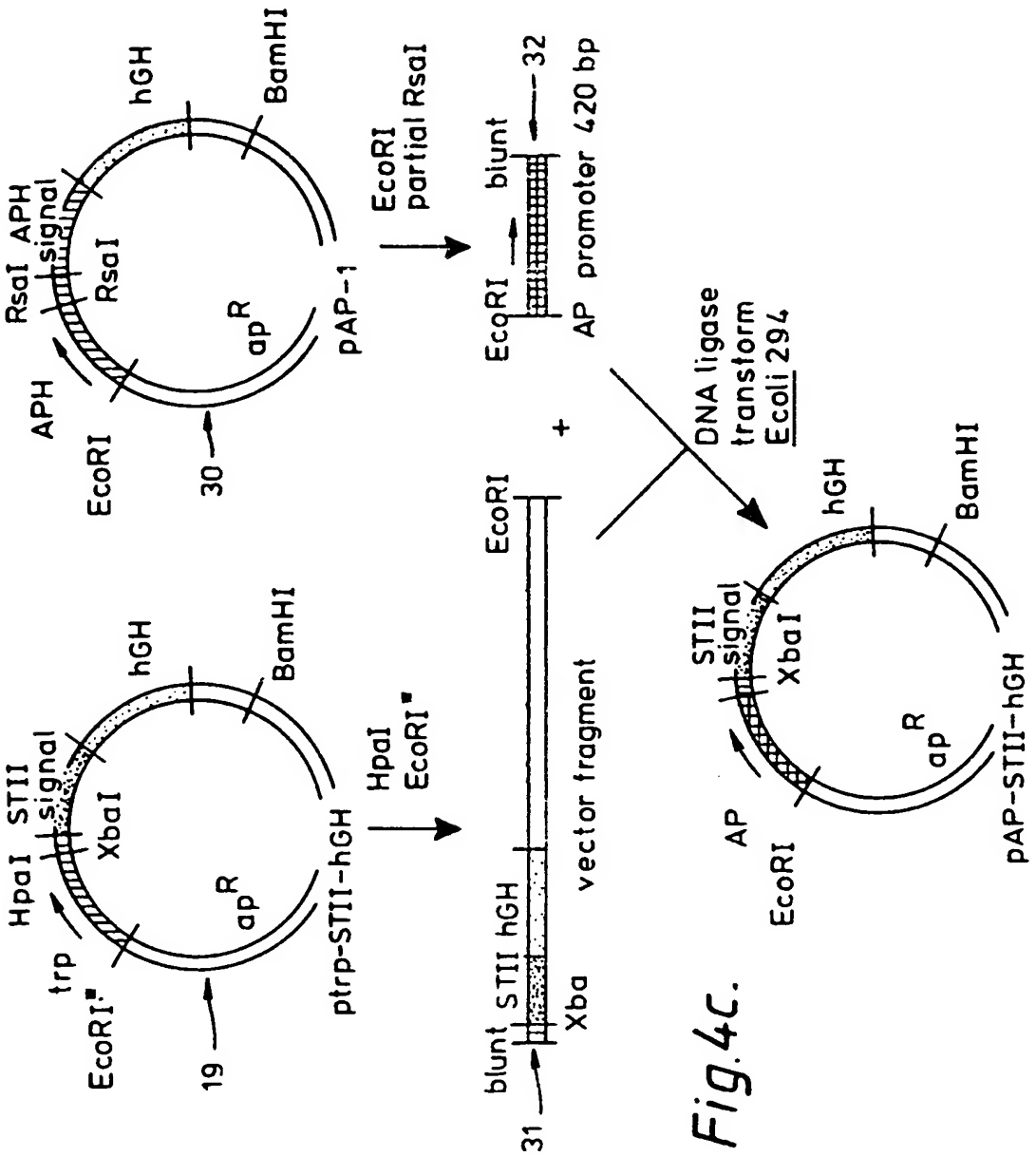

RsaI APH
signal
hGH
BamHI
RsaI
APH
EcoRI
30
ap^R
pAP-1
EcoRI
partial RsaI
EcoRI
blunt
32
AP promoter 420 bp
HpaI STII
signal
XbaI
hGH
BamHI
trp
EcoRI'
19
ap^R
ptrp-STII-hGH
HpaI
EcoRI'
EcoRI
blunt STII hGH
31
Xba
vector fragment
+
DNA ligase
transform
Ecoli 294
STII
signal
XbaI
hGH
BamHI
AP
EcoRI
ap^R
pAP-STII-hGH

Fig.4c.

```
GAATTCAACTTCTCCATACTTTGGATAAGGAAATACAGACATGAAAAATCTCATTGCTGAGTTGTTATTTAAGCTTGCC

CAAAAAGAAGAAGAGTCGAAAGAACTGTGTGCGCAGGTAGAAGCTTTGGAGATTATCGTCACTGCAATGCTTCGCAATA

TGGCGCAAAATGACCAACAGCGGTTGATTGATCAGGTAGAGGGGGCGCTGTACGAGGTAAAGCCCGATGCCAGCATTCC

TGACGACGATACGGAGCTGCTGCGCGATTACGTAAAGAAGTTATTGAAGCATCCTCGTCAGTAAAAAGTTAATCTTTTC

AACAGCTGTCATAAAGTTGTCACGGCCGAGACTTATAGTCGCTTTGTTTTTATTTTTTAATGTATTTGTAACTAGTACG

                                                    -23         -20
              trp S.D.              STII S.D.     met lys lys asn ile ala phe leu leu
CAAGTTCACGTAAAAAGGGTATCTAGAAGGTTGAGGTGATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT
                      xbaI

                 -10                                              1
ala ser met phe val phe ser ile ala thr asn ala tyr ala phe pro thr ile pro leu
GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAT GCC TAT GCA TTC CCA ACT ATA CCA CTA

               10                                            20
ser arg leu phe asp asn ala met leu arg ala his arg leu his gln leu ala phe asp
TCT CGT CTA TTC GAT AAC GCT ATG CTT CGT GCT CAT CGT CTT CAT CAG CTG GCC TTT GAC

               30                                            40
thr tyr gln glu phe glu glu ala tyr ile pro lys glu gln lys tyr ser phe leu gln
ACC TAC CAG GAG TTT GAA GAA GCC TAT ATC CCA AAG GAA CAG AAG TAT TCA TTC CTG CAG
```

Fig. 5.

```
            50                                          60
asn pro gln thr ser leu cys phe ser glu ser ile pro thr pro ser asn arg glu glu
AAC CCC CAG ACC TCC CTC TGT TTC TCA GAG TCT ATT CCG ACA CCC TCC AAC AGG GAG GAA

            70                                          80
thr gln gln lys ser asn leu glu leu leu arg ile ser leu leu leu ile gln ser trp
ACA CAA CAG AAA TCC AAC CTA GAG CTG CTC CGC ATC TCC CTG CTG CTC ATC CAG TCG TGG

            90                                          100
leu glu pro val gln phe leu arg ser val phe ala asn ser leu val tyr gly ala ser
CTG GAG CCC GTG CAG TTC CTC AGG AGT GTC TTC GCC AAC AGC CTA GTG TAC GGC GCC TCT

            110                                         120
asp ser asn val tyr asp leu leu lys asp leu glu glu gly ile gln thr leu met gly
GAC AGC AAC GTC TAT GAC CTC CTA AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG ATG GGG

            130                                         140
arg leu glu asp gly ser pro arg thr gly gln ile phe lys gln thr tyr ser lys phe
AGG CTG GAA GAT GGC AGC CCC CGG ACT GGG CAG ATC TTC AAG CAG ACC TAC AGC AAG TTC

            150                                         160
asp thr asn ser his asn asp asp ala leu leu lys asn tyr gly leu leu tyr cys phe
GAC ACA AAC TCA CAC AAC GAT GAC GCA CTA CTC AAG AAC TAC GGG CTG CTC TAC TGC TTC

            170                                         180
arg lys asp met asp lys val glu thr phe leu arg ile val gln cys arg ser val glu
AGG AAG GAC ATG GAC AAG GTC GAG ACA TTC CTG CGC ATC GTG CAG TGC CGC TCT GTG GAG

            190 191
gly ser cys gly phe AM        ┌pBR322
GGC AGC TGT GGC TTC TAG CTGCCC┌TTAATGCGGTAGTTTATCACAGTT
                              SmaI-Blunt HindIII junction
```

Fig. 5(Cont.)

```
GAATTCAACTTCTCCATACTTTGGATAAGGAAATACAGACATGAAAAATCTCATTGCTGAGTTGTTATTTAAGCTTGCC

CAAAAAGAAGAAGAGTCGAAAGAACTGTGTGCGCAGGTAGAAGCTTTGGAGATTATCGTCACTGCAATGCTTCGCAATA

TGGCGCAAAATGACCAACAGCGGTTGATTGATCAGGTAGAGGGGGCGCTGTACGAGGTAAAGCCCGATGCCAGCATTCC

TGACGACGATACGGAGCTGCTGCGCGATTACGTAAAGAAGTTATTGAAGCATCCTCGTCAGTAAAAAGTTAATCTTTTC

                                                                    AP -   SD
AACAGCTGTCATAAAGTTGTCACGGCCGAGACTTATAGTCGCTTTGTTTTTATTTTTTAATGTATTTGTACATGGAGAA

        -21 -20                                        -10
        met lys gln ser thr ile ala leu ala leu leu pro leu leu phe thr pro val
AATAAA  GTG AAA CAA AGC ACT ATT GCA CTG GCT CTC TTA CCG TTA CTG TTT ACC CCT GTG

              1                                    10
thr lys ala phe pro thr ile pro leu ser arg leu phe asp asn ala met leu arg ala
ACA AAA GCC TTC CCA ACT ATA CCA CTA TCT CGT CTA TTC GAT AAC GCT ATG CTT CGT GCT

         20                                        30
his arg leu his gln leu ala phe asp thr tyr gln glu phe glu glu ala tyr ile pro
CAT CGT CTT CAT CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT GAA GAA GCC TAT ATC CCA

         40                                        50
lys glu gln lys tyr ser phe leu gln asn pro gln thr ser leu cys phe ser glu ser
AAG GAA CAG AAG TAT TCA TTC CTG CAG AAC CCC CAG ACC TCC CTC TGT TTC TCA GAG TCT
```

Fig. 6.

```
        60                                              70
ile pro thr pro ser asn arg glu glu thr gln gln lys ser asn leu glu leu leu arg
ATT CCG ACA CCC TCC AAC AGG GAG GAA ACA CAA CAG AAA TCC AAC CTA GAG CTG CTC CGC

        80                                              90
ile ser leu leu leu ile gln ser trp leu glu pro val gln phe leu arg ser val phe
ATC TCC CTG CTG CTC ATC CAG TCG TGG CTG GAG CCC GTG CAG TTC CTC AGG AGT GTC TTC

        100                                             110
ala asn ser leu val tyr gly ala ser asp ser asn val tyr asp leu leu lys asp leu
GCC AAC AGC CTA GTG TAC GGC GCC TCT GAC AGC AAC GTC TAT GAC CTC CTA AAG GAC CTA

        120                                             130
glu glu gly ile gln thr leu met gly arg leu glu asp gly ser pro arg thr gly gln
GAG GAA GGC ATC CAA ACG CTG ATG GGG AGG CTG GAA GAT GGC AGC CCC CGG ACT GGG CAG

        140                                             150
ile phe lys gln thr tyr ser lys phe asp thr asn ser his asn asp asp ala leu leu
ATC TTC AAG CAG ACC TAC AGC AAG TTC GAC ACA AAC TCA CAC AAC GAT GAC GCA CTA CTC

        160                                             170
lys asn tyr gly leu leu tyr cys phe arg lys asp met asp lys val glu thr phe leu
AAG AAC TAC GGG CTG CTC TAC TGC TTC AGG AAG GAC ATG GAC AAG GTC GAG ACA TTC CTG

        180                                             190 191
arg ile val gln cys arg ser val glu gly ser cys gly phe AM
CGC ATC GTG CAG TGC CGC TCT GTG GAG GGC AGC TGT GGC TTC TAG CTGCCC
```

Fig.6(Cont.)